Identification of Biogenic Volatile Organic Compounds for Improved Border Biosecurity

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Effective border biosecurity is a high priority in New Zealand. A fragile and unique natural ecosystem combined with multiple crop systems, which contribute substantially to the New Zealand economy, make it essential to prevent the establishment of invasive pests. Trade globalisation and increasing tourism have facilitated human-assisted movement of invasive invertebrates, creating a need to improve pest detection in import pathways and at the border. The following works explore a potential new biosecurity inspection and monitoring concept, whereby unwanted, invasive insects may be detected by the biogenic volatile organic compounds (VOCs) they release within contained spaces, such a ship containers.

The brown marmorated stink bug, *Halyomorpha halys* Stål, is an agricultural and urban pest that has become widely established as an invasive species of major concern in the USA and across Europe. This species forms large aggregations when entering diapause, and it is often these aggregations that are found by officials conducting inspections of internationally shipped freight. Stink bug species are known to emit defensive odours, making *H. halys* a suitable candidate as model species for this study.

Undisturbed aggregations of diapausing *H. halys* were found to emit tridecane and *(E)*-2-decenal. Mechanical agitation of diapausing *H. halys* was used to induce emissions of defensive odours, and the full VOC profile was confirmed through GC-MS analysis as: tridecane (41.7 ± 11.8 µg per bug), *(E)*-2-decenal (18.2 ± 4.2 µg), 4-oxo-*(E)*-2-hexenal (15.8 ± 6.3 µg), and dodecane (1.5 ± 0.6 µg). Testing the

role of conspecific bugs on VOC release, it was found that *H. halys* required the presence of another bug as well as mechanical agitation to elicit a defensive odour response. From this, the effect of conspecific defence compounds were individually tested on single *H. halys*. One component, 4-oxo-*(E)*-2-hexenal, was found to cause individual bugs to both move further distances after exposure, and also release their own defensive odour. Thus, the agitation of aggregations, as it might occur during freight shipping, could facilitate an amplification effect for release of odours; were one bug to emit defensive VOCs in an aggregation, more would be likely to emit. This may increase the likelihood of detection of these VOCs within an enclosed space such as shipping containers.

Experiments were performed to simulate the effects of two variables introduced by the act of shipping, ship movement and journey temperature fluctuations, upon aggregations of diapausing *H. halys*. Aggregations exposed to simulated shipping movement, using a 6-axis VS-6577G-B Denso robot arm, were not found to be any more likely to release VOCs than aggregations which remained stationary, nor did it cause any bugs to become mobile. Simulated temperature changes as they would be experienced during a voyage over 26 days from a port in the north-east USA to New Zealand were found to have a significant effect on the mobility of *H. halys*. However, towards the end of the simulated voyage, most *H. halys* died, probably from a lack of food or moisture in the shipping scenario. The high mortality observed in these aggregations prompted the collection of headspace samples from dead *H. halys* over the same time period and experiencing the same temperatures. This revealed that dead and decaying *H. halys* release the full VOC profile of tridecane, *(E)*-2-decenal, 4-oxo-*(E)*-2-hexenal, and dodecane over three weeks, although in smaller quantities than when actively releasing defensive odours.

Theoretical calculations showed that the GC-MS analytical method combined with active sampling volatile collection traps was not sensitive enough to detect volatiles released by aggregations of living, dead, or combined *H. halys* within a 20 foot (38,000 l) shipping container. However, there are more sensitive technologies available which can detect VOCs to the parts per trillion level, which would be

capable of detecting the expected VOCs concentrations associated with the presence of *H. halys* in shipping containers.

Keywords: Border biosecurity; pathway risk management; invasive invertebrates; brown marmorated stink bug; *Halyomorpha halys*; Pentatomidae; detection; gas chromatography-mass spectrometry; volatile organic compounds; tridecane; *(E)*-2-decenal; 4-oxo-*(E)*-2-hexenal; dodecane.

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"Live as if you were to die tomorrow. Learn as if you were to live forever."

— Mahatma Gandhi

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"It's a dangerous business, Frodo, going out your door. You step onto the road, and if you don't keep your feet, there's no knowing where you might be swept off to."

— J.R.R. Tolkien, The Lord of the Rings

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Chapter 1

An Introduction to New Zealand Biosecurity and an Overview of *Halyomorpha halys* (Hemiptera: Pentatomidae) as a Biosecurity Threat

1.1 New Zealand Biosecurity

The geographical isolation of New Zealand gives it a highly unique and fragile ecology. Aside from the rare natural ecosystems found here which require protection, there are controlled systems of pastoral, crop and orchard agriculture which are a large part of the New Zealand economy (Biosecurity Council, 2003). Effective biosecurity is therefore essential to protect such systems from invasive species. Human-assisted movement of invasive invertebrates across borders has become a big issue with globalisation, and climate change is also facilitating the establishment of alien species in new regions. New Zealand has long-standing and in-depth strategies to cope with agricultural biosecurity, beginning with risk assessment, pathway risk management and stretching to long term management solutions should a species establish (Goldson, Barratt, & Armstrong, 2016). The safest, most cost-effective strategy is to have efficient pre-border and border biosecurity procedures. Incoming passenger and mail traffic is monitored through the use of declaration cards for information pre-border, with further x-ray machines and sniffer dogs located at the border. However, the biggest challenge faced by border biosecurity is the large quantities of diverse material arriving as freight in sea containers of which there are approximately 600,000 entering New Zealand every year (Goldson, Barratt, & Armstrong, 2016). Pre-border information about this kind of cargo is collected via risk profiling, and bills of lading, which, while not preventative in themselves, indicate high risk containers. Once landed in New Zealand, containers are stored at transitional facilities and visually inspected before cargo release. These procedures have indicated that hitchhiker pests are common on this pathway: a survey of close inspections of air freight containers has revealed 13.2 % of inspected goods

contained contaminants requiring quarantine (Gadgil, Bulman & Glassey, 2002). Despite these measures, not all containers can be closely inspected given the amount of traffic entering New Zealand, and incursions by alien invertebrates have continued to occur (Brockerhoff & Liebhold, 2017). New technologies must always be considered for the detection of biosecurity threats entering through the shipping pathway, as alien invertebrates present the additional challenge of being mobile and readily escape prior to or during inspection. Ants offer a good example of this issue, as nests of different species have often been found at ports and transitional facilities in New Zealand. These include *Paratrechina longicornis* Latreille, commonly called crazy ant due to its long legs and erratic movements, *Solenopsis geminate* Fabricius and *Solenopsis Invicta* Buren, the tropical fire ant and red imported fire ant respectively. These have been identified amongst the seven most widespread invasive ant species in the world (Holway, Lach, Suarez, Tsutsui, & Case, 2002; Ness & Bronstein, 2004), and all are considered high risk for New Zealand border biosecurity (Harris et al., 2005). *Paratrechina longicornis* is frequently intercepted at the border in containers of fresh produce, wood, cut flowers and items from personal storage. Most interceptions have been of worker ants, however, more significantly nests with queens have also been discovered. In 2003, three nests were found in shipments arriving at Port Tauranga (Harris et al., 2005). Since 2001, there have been three incursions of *S. invicta* in New Zealand, with two nests being found in border control areas near ports, and one further inland. The current status of *S. invicta* in New Zealand is eradicated, but there is still concern, as it has shown that it can colonise here. Workers of *S. geminata* also are frequently intercepted at the border, and occasionally nests with queens are found, on a range of imported commodities, mostly dominated by fresh produce and nursery stock (Harris et al., 2005). The coccinellid *Harmonia axyridis* Pallas, commonly referred to as the harlequin ladybird or multi-coloured Asian ladybird, is still considered a high biosecurity risk, although there are now established populations on the North Island of New Zealand. *Harmonia axyridis* is predatory, predominantly feeding on aphid species, which are worldwide crop pests, and has therefore been introduced as a biocontrol agent across four continents. A variety of crops, e.g. apples, soybean, citrus fruits, maize, tobacco and cotton have benefited from

this effective pest control method (Brown et al., 2011; Cai, Koziel, & O'Neal, 2007). It has also established itself in numerous countries where it was not intentionally introduced, including Canada, Brazil, the UK and South Africa (Brown et al., 2011). Despite its success as a biological control agent, *H. axyridis* is equally considered an invasive pest because of its impacts on non-target species. *Harmonia axyridis* had previously been intercepted at the New Zealand border on imported fruits, and considered a risk on the container pathway (Tyson, Rainey, Breach, & Toy, 2009). However, in 2016, an established population was confirmed in Auckland, and another has since been reported in the Bay of Plenty.

These cases show that border inspections of the high risk container pathway needs improvement. The work described in this thesis provides an example of a new potential biosecurity inspection and monitoring concept, whereby alien invertebrate species may be detected by the biogenic volatile organic compounds (VOCs) released by all organisms. This contribution demonstrates how knowledge of those compounds released by a potential threat, and the behaviours surrounding VOC release, can well lay a solid foundation for this concept.

High on New Zealand's Ministry for Primary Industries (MPI) priorities within the border biosecurity sector is *Halyomorpha halys* Stål, commonly known as the brown marmorated stink bug. This highly invasive and destructive crop pest releases a distinct odour when alarmed (Weber et al., 2017). For this reason, it is an excellent candidate to model the concept of VOC detection on.

1.2 *Halyomorpha halys* pest status

Originally from China, Korea, Japan, and Taiwan, this pest species has established in 44 states of the USA, four provinces in Canada, and is spreading through European countries (http://www.stopbmsb.org, last accessed 15th November 2017). *Halyomorpha halys* feeds on numerous crops of economic significance. Preferred host plants include apple, peach, grape, and soybean. Costly damage has been reported within *H. halys'* native Asian ranges where 106 hosts are known (Lee, Short, Joseph, Bergh, & Leskey, 2013). Severe crop damage occurring in at least nine US

states is well documented, with the Eastern states taking the brunt. Extensive research has been done, and is still underway, on the management of this established species, predominantly in the USA. In China, *H. halys* can be multivoltine, whereas in the USA it is uni- or bivoltine (Hoffman, 1931; Nielsen, Hamilton, & Matadha, 2008). Despite decreased generations per year, *H. halys* populations hit a high in 2010 in the USA and caused US$37 million damage in apple crops alone (Rice et al., 2014).

Halyomorpha halys aggregates to overwinter; in natural habitats this occurs on trees, either in holes or under loose bark, and at high elevations (Lee et al., 2013). In urban areas, *H. halys* enters dwellings and aggregates in cool, dry areas such as attics and wall insulations (Leskey, Hamilton, et al., 2012). This causes a nuisance as they emit a foul odour when disturbed, also causing staining on walls and furnishings. This species is therefore viewed as both a severe crop and an urban nuisance pest.

1.2.1 *Halyomorpha halys* as a surveillance issue and current monitoring

For countries which have yet to have this pest arrive, monitoring within the biosecurity sector is of great import. This is especially in light of research confirming that the US populations originated from a single incursion from Beijing, which goes to show how important it is to prevent post-border incursions (Xu, Fonseca, Hamilton, Hoelmer, & Nielsen, 2014). New Zealand and Australia are therefore especially concerned about keeping this pest out; indeed, their geographic isolation makes it more realistic to sustain a prevention strategy than countries in mainland Europe which are proximal to already-invaded regions. Nonetheless, New Zealand and parts of Australia fall into latitudes which would be climactically suitable for the establishment of *H. halys* (Aigner & Kuhar, 2016; Kriticos et al., 2017; Zhu, Bu, Gao, & Liu, 2012). Unfortunately, these Southern Hemisphere countries have experienced a noticeable increase in *H. halys* interceptions over recent years (Duthie, 2012). Within every year, there is an increase in interceptions between October and March, because these are the months when *H. halys* undergoes Northern Hemisphere diapause. In terms of international spread, these overwintering aggregations can occur within personal effects and goods to be shipped, vehicle exports, and within the shipping containers themselves. In the aforementioned Southern Hemisphere

countries, there is thus a concerted monitoring effort to prevent *H. halys* from crossing borders in airports, mail rooms, and sea ports. Sniffer dogs can be used in the former two locations, but have been deemed unsuitable for use in sea port. Suitable, high-risk freight, such as vehicles must either be fumigated with methyl bromide, or heat treated at 60°C to kill any *H. halys* hitchhikers before entering these countries (Department of Agriculture, 2015; Thompson, 2014); the lethal high for *H. halys* was found to be >50°C (Aigner & Kuhar, 2016).

As the most intensive surveillance challenges occur during *H. halys'* Northern Hemisphere diapause period, a focus on researching this species in diapause is logically the most appropriate.

1.3 *Halyomorpha halys* in diapause

1.3.1 Triggers of *Halyomorpha halys* diapause onset

Halyomorpha halys aggregate and go into facultative diapause when overwintering to slow their metabolism and prolong their lives (Saulich & Musolin, 2012). The associated fasting is particularly beneficial during the winter months when food is scarce. There is no definitively reported trigger for the start of *H. halys* diapause, although lower temperatures and shortening days are thought to be the main contributors (Niva & Takeda, 2002, 2003). In their native range, *H. halys* emerge from overwintering any time from late March onwards when ambient temperatures are >10°C (Lee et al., 2013; Qin, 1990). In general, it has been reported that *H. halys* are chill intolerant, and therefore die before reaching their freezing point (Cira et al., 2016). Hence the preference for aggregating in buildings in those regions which reach more extreme cold temperatures than occur their native range (i.e. regions of North America). The species also exhibits a preference for darkness, which may be an evolved response to direct sunlight and associated fluctuating temperatures (Toyama, Ihara, & Yaginuma, 2011). Sunlight can cause desiccation of the overwintering bugs, and increases in temperature would speed up their metabolism. Further, the preference for narrow spaces by both the reproductive and diapause stages may be a mechanism for avoiding predators such as birds, and abiotic factors such as rain and wind.

Females enter diapause when reproductively immature and this is required if they are to subsequently reach sexual maturity (Taylor, Coffey, Hamby, & Dively, 2017). Taylor et al. (2017) found that a minimum diapause period of 7 weeks is required before *H. halys* can terminate diapause with full fecundity. Termination of diapause is triggered by combinations of temperature increases, increasing photoperiods and depletion of metabolic resources levels (Funayama, 2012; Kiritani, 2007). Nielson et al. (2016) have established that a critical photoperiod of 13.5:10.5 L:D is required for diapause termination in *H. halys* in the US.

1.3.2 Aggregative behaviour of overwintering *Halyomorpha halys*

In natural, native landscapes, *H. halys* is reported to overwinter under loose tree bark, and in cliff outcrops, showing a preference for elevation, and dark, narrow spaces (Watanabe, 1994). Lee et al. (2014) aimed to further characterise natural *H. halys* diapausing sites in the invaded US range, focussing on those dead trees where diapausing bugs may be found. Over two winter seasons in four locations within Maryland and West Virginia, the researchers inspected dead trees for the presence of diapausing *H. halys*. The ensuing data were used to characterise which species, size, and arrangement of tree was most likely to be used as diapause sites. The larger (diameter >19 cm), standing dead trees comprising elm, tree of heaven, locust, and oak were found to be preferred by *H. halys*. Inkley (2012) was the first to publish a full analysis of a residential *H. halys* infestation. He sampled from his own house, and made a concerted effort to collect every stink bug from the first and second levels of the house using a vacuum cleaner. Over a six month period (1st January – 30th June 2011) these were counted daily and then immediately destroyed. For the first five months, when *H. halys* were present during their diapause period, 25 or more stink bugs were collected per day on 56% of days, and on 21% of the days more than 100 bugs were collected per day. The author believed the severity of his home infestation was due to many preferential factors for *H. halys*. The house structure had an unfinished basement and the attic areas made for good entrances during autumn. Further, the location was rural and close to deciduous forests and cropland containing host plants.

Toyama et al. (2011) carried out observational experiments on *H. halys'* photo response preferences, focussing on the context of overwintering behaviour. They found that diapausing adults will choose dark over light refuges. They also compared three temperature settings during the experiments (14, 18 and 24°C) but these had little to no effect on the diapausing bugs. However, non-diapausing bugs at the higher temperatures were found to be less likely to be in the dark shelter. This was thought to be because of an increase in activity in the warmer environments rather than a change in photo-preference. Another study investigated the role of antennae in short range interaction between aggregating stink bugs (Toyama, Ihara, & Yaginuma, 2006). The only variable that appeared to reduce the tendency to aggregate was the removal of antennae; this had a far greater effect than changing light and temperature conditions. These combined studies led to a division of *H. halys*' overwintering aggregation behaviours into two theoretical stages: long distance and short distance aggregation. The bugs arrive in a vicinity through preferential site choices. Once at those sites, the bugs use their antennae to pick up some unknown aggregative signal.

1.3.3 Management of diapausing *Halyomorpha halys* populations

A male-produced aggregation pheromone from *H. halys* was identified and synthesised for use in field trapping (Khrimian et al., 2014). The specific, two-component pheromone attracts adults and nymphs of both sexes during the feeding and reproduction seasons. The pheromone is made up of (3S, 6S, 7R, 10S)-10, 11-epoxy-1-bisabolen-3-ol and (3R, 6S, 7R, 10S)-10, 11-epoxy-1-bisabolen-3-ol at a natural ratio of 3.5:1. This pheromone was tested in combination with methyl (E,E,Z)-2,4,6-decatrienoate (MDT) (Weber, Leskey, Walsh, & Khrimian, 2014). MDT is an aggregation pheromone of the Asian stink bug species, *Plautia stali* Scott, previously found to attract late season *H. halys*. Traps containing lures combining both were 1.9-3.2 times more likely to catch adult *H. halys* than pheromone-only lures. MDT lures were not significantly effective in catching adult *H. halys* in the growing season but became increasingly attractive in autumn. This led to a season-long study on the success rate of traps containing different compositions of the pheromone and synergists (Leskey, Agnello, et al., 2015). Pheromone-containing traps were highly successful when adults emerged from overwintering

through to late season. However, the bugs became unresponsive to the same pheromonal stimuli in the autumn, when they were heading to their overwintering sites. A study also found that stink bugs overwintering in man-made dwellings did not respond to indoor pheromone traps (Morrison et al., 2017a). In heated buildings, pheromone pyramid traps were trapping on average 8% of bugs which had been identified visually as active during winter months within the building. This figure increased to 20% as spring set in, presumably when bugs were taking queues to begin emerging. In short, it would seem that stink bugs which were in diapause do not respond in the same way as active bugs.

1.4 Objectives of this thesis

The following objectives will be applied to *Halyomorpha halys* as a model species to test VOC-based methods with which to determine the presence of invasive species in enclosed spaces. Such capability has significant potential to contribute to general border biosecurity procedures.

1) Identifying VOC patterns and quantities released by diapausing *Halyomorpha halys.*
2) Characterising the relationship between VOCs released and behavioural cues for defensive emissions of diapausing *Halyomorpha halys*.
3) Applying knowledge from 1) and 2) to a practical situation, i.e. simulating a biosecurity situation to determine whether VOC release and behavioural cues apply.
4) Optimising sensitive analytical collection and analysis techniques for VOC profile, and applying them to a detection limit for large, contained volumes.

Halyomorpha halys is the nominated model species as it has been shown to be an adept hitchhiker, which is capable of establishing in New Zealand, and would be likely to cause significant economic losses upon establishment. This is also a species which has a high likelihood of producing an identifiable VOC profile when agitated, due to its defensive odour responses. There is also the possibility of a VOC signature when bugs are undisturbed.

A study on the proficiency of detector canines to locate diapausing *H. halys* was trialled in both laboratory and semi-field situations (Lee et al., 2014). Over 84% accuracy was observed by two detector canines in identifying positive *H. halys* locations and ignoring blank controls. This suggests that there are indeed VOCs with which to detect *H. halys*, however high sensitivity will be needed to detect such signals.

Chapter 2

Identification of Volatiles Released by Diapausing Brown Marmorated Stink Bug, *Halyomorpha halys* (Hemiptera: Pentatomidae)

(As submitted for publication to PlosOne: Laura J. Nixon, William R. Morrison, Kevin B. Rice, Eckehard G. Brockerhoff, Tracy C. Leskey, Filadelfo Guzman, Ashot Khrimian, Stephen Goldson, Michael Rostás)

2.1 Introduction

Halyomorpha halys Stål, commonly known as the brown marmorated stink bug, has emerged as a severe agricultural and urban pest in the USA (Rice et al., 2014). As of November 2017, *H. halys* have successfully invaded and established in 44 states of the USA, Canada, and nine European countries (Italy, France, Hungary, Switzerland, Germany, Liechtenstein, Greece, Serbia, and Romania), with recent incursions reported in Russia, Georgia, and Bulgaria (Gapon, 2016; Haye et al., 2015; Milonas & Partsinevelos, 2014; Rice et al., 2014; Simov, 2016; Valentin, Nielsen, Wiman, Lee, & Fonseca, 2017; Vetek, Papp, Haltrich, & Redei, 2014). Europe's temperate climate is suitable for its success; the models developed by Zhu et al. (2012) identify regions within the latitudes 30° - 50° as high risk from *H. halys* invasion. The species has also recently been reported in Chile, its first Southern Hemisphere invasion (Faúndez & Rider, 2017).

Like many heteropterans, *H. halys* spends the winter months in diapause (Lee et al., 2013). In the USA, the bugs begin to disperse in late summer to overwintering sites. Large numbers aggregate at suitable sites and go into diapause from early to mid-October with spring emergence commencing in April. Typical overwintering sites include dry, protected structures including human-made dwellings, beneath the bark of dead and standing trees (Lee et al., 2014), as well as dry, elevated locations (Lee et al., 2013). This behaviour causes problems for border biosecurity in other countries, as *H. halys* is

an adept hitchhiker and overwintering aggregations can be exported in personal effects or vehicle shipments (Aigner & Kuhar, 2016). Conditions caused by shipment, such as temperature increases, photoperiod shifts, and constant movements, have the potential to disrupt the bugs' diapausing state. This is particularly problematic, because pheromone-baited traps that were developed for monitoring *H. halys* during the growing season are ineffective against overwintering populations (Morrison et al., 2017a). The significance of diapausing *H. halys* as a biosecurity risk to the international community makes it prudent to investigate alternative measures to detect *H. halys*, including the chemical emissions and behaviours of this species in diapause and when diapause becomes disrupted. If a method for detection of emissions of volatiles from *H. halys* could be developed, then aggregations of *H. halys* in international freight shipments could be detected and treated, thereby preventing arrival and establishment post-border. Volatile organic compounds (VOCs) can be continuously released by organisms. Moreover, many heteropterans also release defensive VOCs usually consisting of tridecane and at least one *(E)*-2-aldehyde, whether it be the 6-, 8-, or 10-carbon aldehyde. Aldehydes are the odour compounds for which "stink" bugs are named (Millar, 2005). The question of whether diapausing populations of *H. halys* release detectable VOCs, or show such a defensive reaction, has not been previously studied. Thus, it is necessary to first establish if VOCs can be detected from aggregations of diapausing populations, and whether this defensive reaction is exhibited by diapausing bugs. This contribution discusses an emissions profile found from diapausing and diapause-disrupted adults of *H. halys*, both at rest and when agitated.

2.2 Methods and materials

2.2.1 Field samples of *Halyomorpha Halys*

For the purposes of this study, cohorts of naturally diapausing wild adult *H. halys* were used. Simulated overwintering sites, i.e., wooden shelters, as described by Bergh et al. (2017), were deployed in September 2015, just prior to dispersal to potential overwintering sites. Shelters were deployed at an organic farm (Redbud Farm) in Inwood, WV (39°23'41.49"N, 78° 4'39.84"W), at Mount Weather, VA

(39° 3'43.77"N, 77°53'29.65"W), Boonsboro, MD (39°30'20.55"N, 77°44'34.95"W), and Gerrardstown, WV (39°24'22.19"N, 78° 5'54.68"W).

Thereafter, the shelters were collected in early November 2015 after diapause onset and maintained in a dark unheated shed at USDA-ARS Kearneysville, WV (39°21'18.69"N, 77°52'40.71"W), from November 2015 to March 2016 under ambient temperature conditions (mean ± *SE*: 6.3 ± 0.03°C). Adults were then utilised based on two relevant biological regimes. These included inactive, diapausing individuals that remained aggregated and diapausing individuals that had become active, i.e., foraging, feeding, and/or reproducing, due to favourable abiotic conditions. To evaluate these two potential conditions, adults described as "diapausing" were taken directly from shelters retrieved from the unheated shed and immediately exposed to the relevant experimental conditions. Adults referred to as "diapause-disrupted" were also taken from shelters in the unheated shed, but subsequently exposed to long photoperiod (16:8. L:D), higher temperatures (24.4 ± 0.2°C), and supplied with food (carrots, sundried tomatoes, and sunflower seeds) *ad libitum* for at least 2 weeks, to begin to break diapause and induce foraging behavior.

2.2.2 VOC emission by diapausing *Halyomorpha halys*

To establish whether there is a sufficient VOC emission profile released by non-agitated bugs, naturally diapausing aggregations of *H. halys* were resettled from the wooden shelters into metal shelters (*n*=7) and left outside in a darkened shed under ambient temperature and relative humidity. The metal shelters (18 × 22 × 20.5 cm, H × L × W) mimicked the design of the already established wooden shelters as overwintering locations for *H. halys* (15). A total of 10, ~2 mm wide metal sheets (22 × 19.5 cm, L × W) were spaced 9 mm apart in half of the space available within the box and affixed in place with three screws running the length of the box and a series of nuts. Two metal lips on the shelter box overhung the metal sheets by 1.1 cm to prevent the internal assembly from loosening (Figures 1A and B).

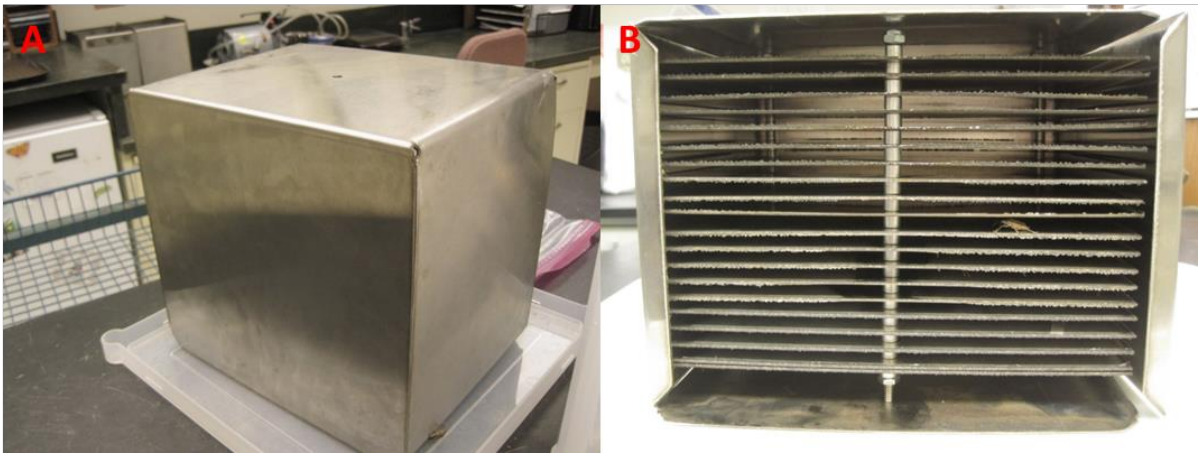

Figure 2.1 A) Photograph of metal sampling box exterior. B) Photograph of metal sampling box interior. Credit: Torri Hancock (USDA-ARS, AFRS).

The resettling process was conducted by taking 68 adult *H. halys* (2.4M: 1F, this ratio was taken from preliminary observations of wild *H. halys* settling into overwintering shelters) from the diapausing populations in the wooden shelters and placing them in cages containing the metal shelter. This cage was placed in a temperature controlled room (24.4 ± 0.2°C) with light exposure for 12 hr; this allowed the bugs to become mobile enough to crawl up into the metal shelter, but was not extended enough to disrupt diapause. The critical cue to terminate *H. halys* diapause has been shown to be 13.5 hr daylight, this needs to be over an extended period of time to trigger any real disruptions (Nielsen et al., 2016; Nielsen et al., 2017). The metal shelters containing populations of *H. halys* were then maintained in a dark outdoor shed under ambient temperature conditions (6.3 ± 0.03°C) for seven days before VOC sampling was performed. This procedure caused minimal disruption to the bugs.

Resettling of the adults into the metal shelters was necessary to eliminate additional background chemical noise, which was an inherent problem for the wooden shelters in which overwintering *H. halys* are normally maintained. The headspace of the metal shelters, containing *H. halys* that had not undergone any mechanical agitation, were sampled using two methods. First, headspace compounds were collected with a portable battery-operated air pump (PAS-500, Spectrex, CA, USA). Air from the shelters was pulled through a volatile collection trap (VCT) containing 30 mg of Super-Q (Analytical Research Systems, FL, USA) at a rate of 400 ml/min for 2 hr. The VOCs were extracted from VCTs using

250 µl of dichloromethane (DCM). Secondly, solid phase micro-extraction (SPME) headspace samples were collected using 100 µm polydimethylsiloxane (PDMS) fibers with a sampling time of 18 hr. PDMS fibers were chosen as they are recommended for sampling volatile compounds with molecular weight 60 – 275; the previously mentioned defensive compounds fall within this weight range. Fibers were conditioned at 230 °C in a GC injection port for 15 min prior to sampling. Blank control samples were taken from an empty metal shelter using both sampling techniques, and analysed alongside the relevant samples to eliminate background volatiles.

2.2.3 Olfactory detection of mechanically agitated *Halyomorpha halys*

We evaluated whether the disposition to emit VOCs differed depending on the bugs' biological state. To cause a significant level of disturbance, diapausing and diapause-disrupted *H. halys* were held in groups of three in 36 ml glass tubes and shaken vigorously by hand for 1 min. This procedure was chosen as a first step to obtain the highest probability of releasing VOCs and as a prerequisite for further experiments that will establish VOC emission under conditions experienced during freight transportation. After 1 min, the experimenter determined if odours were detectable by human olfaction; the same experimenter was involved in this procedure to eliminate observer variation. A total of 25 replicates were completed for each bug condition, and a chi-squared test for independence was performed to assess *H. halys* defensive response in relation to diapause state.

2.2.4 VOCs from mechanically agitated *Halyomorpha halys*

Some mechanically agitated groups of *H. halys* were found to release defensive compounds during the olfactory detection experiment. Where this occurred, representative headspace samples were taken from both diapausing (n=8) and diapause-disrupted (n=6) *H. halys*. Headspace compounds were collected using the VCT method described above, with air from the 36 ml glass tube containing three bugs being sampled for 10 min. The VOCs were extracted from VCTs using 250 µl of DCM with 200 ng/µl tetralin (Sigma-Aldrich, Australia) as an internal standard. A control blank was taken using the same apparatus and extraction technique.

2.2.5 Chemical standards

Quantitative calibration standards of 2, 10, 20, 100, and 200 ng/µl were made from *(E)*-2-octenal, *(E)*-2-decenal, and tridecane (all >94%, Sigma-Aldrich, Australia) diluted using DCM. All calibration standards also contained tetralin (IS) added from a 10 µg/µl stock. *(E)*-2-octenal, *(E)*-2-decenal, and tridecane quantities from *H. halys* headspace samples were calculated using calibration linear equations. For calibration data see Appendix A (A.1). 4-Oxo-(*E*)-2-hexenal was prepared from 2-ethylfuran following Moreira and Millar (2005). To determine whether differences in defensive compound quantity between diapausing and diapause-disrupted *H. halys* were significant, Shapiro-Wilk tests for normality followed by non-parametric Mann-Whitney U two-tailed tests were performed.

2.2.6 Gas chromatography – mass spectrometry

Gas chromatography – mass spectrometry (GC-MS) analysis was performed on an Agilent Technologies 7890A gas chromatograph coupled with 5975c mass selective detector with an HP-5MS column (30 m x 0.25 mm I.D. x 0.25 µm film), and He as an inert gas (located at USDA-ARS, Beltsville, MD). The spectra were obtained in electron-impact (EI) ionization mode at 70 eV. Splitless injections of 1 µl at an injection temperature of 250°C using Agilent Technologies 7683B autoinjector and 7683 autosampler. The SPME injection sampling time was 3 min. The GC was operated at a column flow of 0.9 ml/min. The temperature program started at 40°C for 7 min, followed by ramping 6°C/min until a final temperature of 230°C was reached and held for 5 min. The mass spectrometer was simultaneously run in total ion count mode, with a scanning range 25-550 *m/z*, and selected ion mode, detecting ions at 29, 41 and 55 *m/z* for trans-2-octenal, 43, 55, and 70 *m/z* for trans-2-decenal, and at 43, 57, and 71 *m/z* for tridecane. For samples and standards containing tetralin, ions at 91, 104, and 132 *m/z* were also detected.

2.3 Results and discussion

For access to all chromatographic data see Appendix C.

2.3.1 Compounds detected from aggregations of diapausing *Halyomorpha halys*

Headspace samples collected from undisturbed metal shelters (*n*=7) using trapping filters, were found to contain predominantly tridecane (89.7 ± 6.7% abundance of total compounds detected) in all samples; *(E)*-2-decenal (0.9 ± 0.9%) was detected in one sample, and decanal (9.4 ± 6.8%) in three samples. When SPME fibers were used, all samples contained tridecane (88.6 ± 3.0%), and six contained *(E)*-2-decenal (7.4 ± 2.3%). This suggests that aggregations of diapausing *H. halys* produce, and possibly passively leak, these compounds over time even in the absence of any disturbance (Harris, Abubeker, Yu, Leskey, & Zhang, 2015). This is supported by the findings of Baldwin et al. (2014), who reported that 70% of the total volatiles emitted by active season, non-agitated adult *H. halys* consisted of tridecane and *(E)*-2-decenal. The minor compounds that were detected in our samples, all at <5% abundance, were dodecane (2 samples), decanal (4 samples), *(E)*-2-decenyl acetate (1 sample), a 13C unknown (2 samples), and pentadecane (1 sample). This profile is not specific to *H. halys*, as tridecane is a commonly found VOC, reported in headspaces of treated wood, floral scent mixture, and numerous insect species (Aldrich, 1988; Schiestl, 2010; Vichi et al., 2007). All other VOCs were found inconsistently in low abundance.

2.3.2 Effect of physiological state on VOC emission

The results from the agitation tests showed that diapausing and diapause-disrupted groups both released defensive odour but not 100% of the time in response to the same mechanical agitation. Further, there were differences between the two groups of bugs: 72% of the diapausing groups released the odour, versus 40% of the diapause-disrupted groups. Thus, diapause significantly affected *H. halys'* defensive odour response ($X^2 = 5.195$, $df = 1$, $p = 0.023$). A possible explanation for the higher responsiveness amongst the diapausing bugs could be related to a lack of alternative defensive options; whereas, diapause-disrupted bugs have greater mobility potential (Lee & Leskey, 2015)

enabling them to escape before resorting to chemical defenses. Pentatomids have been shown to prioritize mobility as an escape strategy over the use of defensive chemical response to tactile agitation (Krall, Bartelt, Lewis, & Whitman, 1999). From a practical point of view, the observation that diapausing *H. halys* have been shown to release defensive VOCs indicates a potential for chemical detection of large aggregations for the purpose of detection and interception purposes in trade pathways.

The VOC blends from diapausing and diapause-disrupted bugs were very similar (Table 1). The predominant component, in terms of abundance, emitted by both groups in response to agitation was tridecane, with three minor components consisting of other *n*-alkanes (C_{10} – C_{14}) (Table 1). This homologous series has been commonly reported as a primary component of defensive secretions in pentatomid species, with tridecane predominating. In general, *n*-alkanes are common within heteropteran scent glands, although most are odourless, tridecane has been reported to act as a defensive fumigant (Aldrich, 1988; Gunawardena & Herath, 2017; Mohekar, Osborne, Wiman, Walton, & Tomasino, 2017).

Aldehydes produced by stink bugs have been well documented ever since Tsuyuki et al. (1965) confirmed them as major components produced by both pentatomids and coreids. Of these compounds *(E)*-2-hexenal, *(E)*-2-octenal, and *(E)*-2-decenal are the most commonly reported within pentatomids. Typically, *(E)*-2-decenal appears as a major component alongside tridecane (da Silva et al., 2015). Of these, *(E)*-2-decenal was the only compound consistently released by agitated *H. halys*. *(E)*-2-octenal was detected in less than half of the samples as a minor component, and *(E)*-2-hexenal found not at all, similarly described by Aldrich (1988).

The volatile 4-oxo-*(E)*-2-hexenal was identified by GC-MS (Figure 2.2) and detected as a major compound in all agitated samples. The retention time and the mass spectrum of the early eluting peak at 9.58 min were identical to those of the synthetic standard (Figure 2.3). Both C_6 and C_8 4-oxo-*(E)*-2-alkenals are common within nymphal and adult Heteroptera secretions (Borges & Aldrich, 1992). A minor component detected in four samples total was *(E)*-2-decenyl acetate.

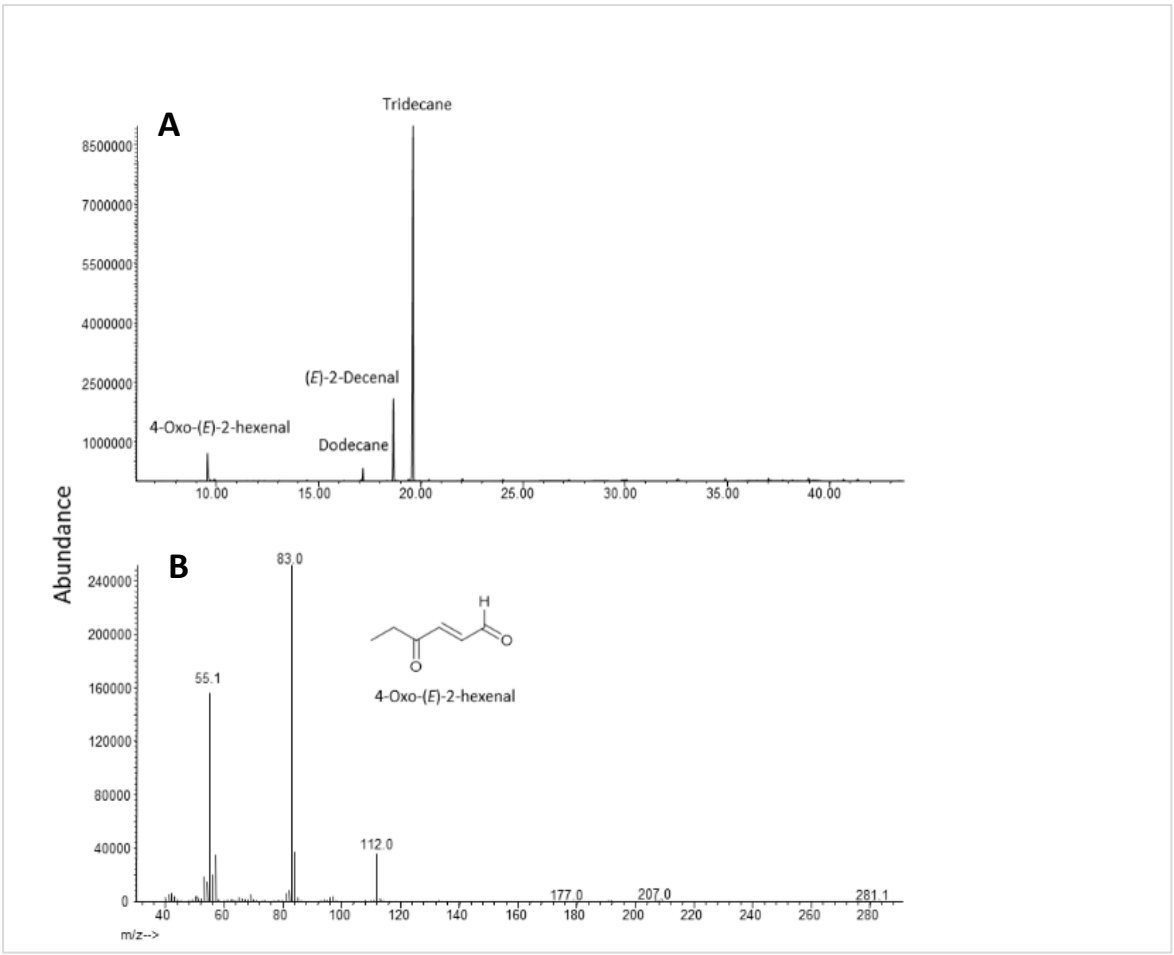

Figure 2.2 **A) GC-MS total ion chromatogram of aeration extract in DCM collected from a group of 10 agitated, diapausing *Halyomorpha halys* on a HP-5MS. B) Mass spectrum of the peak at 9.58 min identified as 4-oxo-(*E*)-2-hexenal. Compounds were identified by comparing GC retention times and mass spectra with those of standards (see also Figure 2.3).**

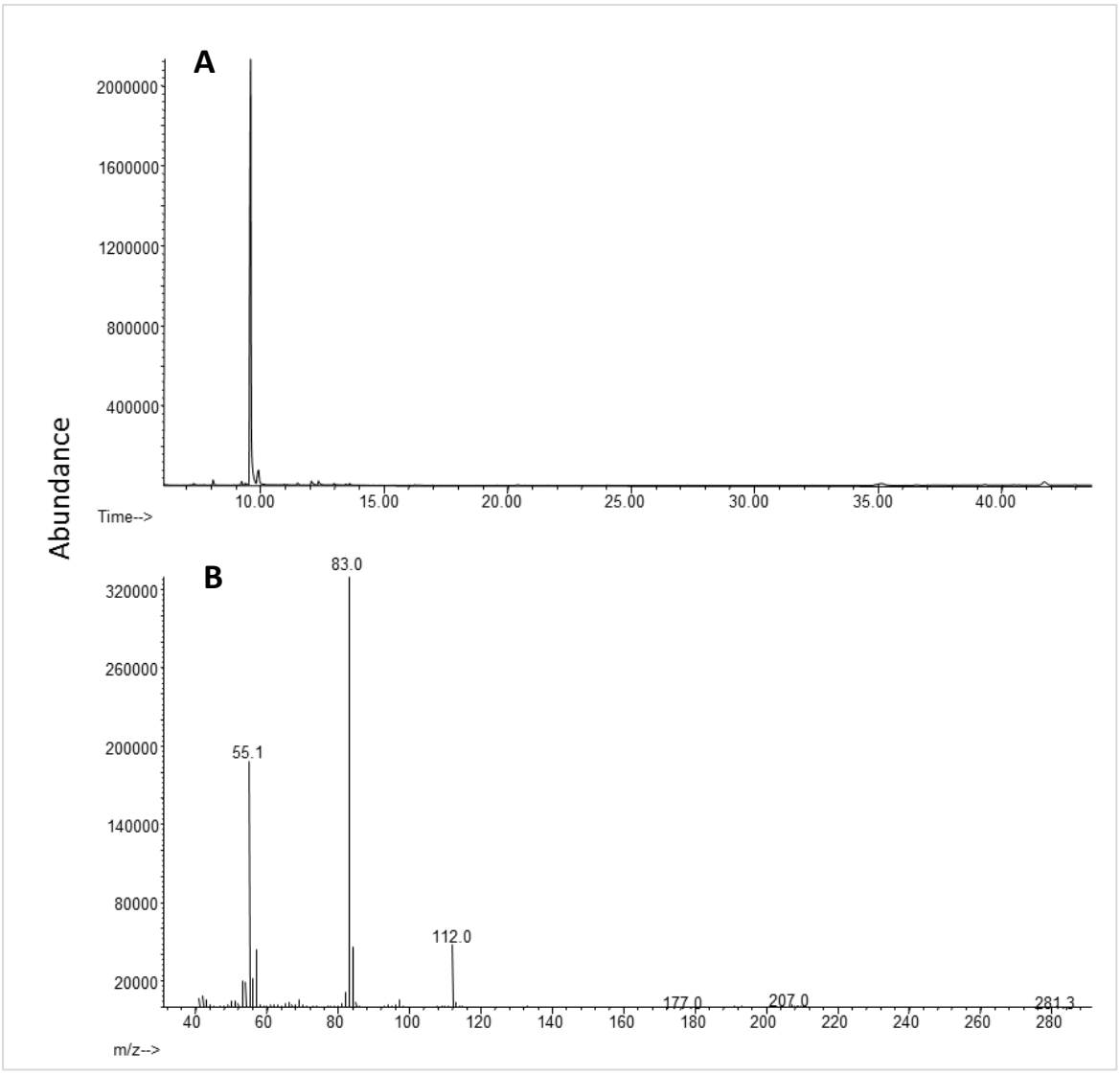

Figure 2.3 A) GC-MS total ion chromatogram and B) mass spectrum of synthetic as 4-oxo-(*E*)-2-hexenal.

The previously known and expected defensive compounds, *(E)*-2-octenal, *(E)*-2-decenal, and tridecane, were quantified (Table 2.1). The amounts calculated to be released per adult of all three compounds did not follow a normal statistical distribution and there were no significant differences in absolute amounts between diapausing and diapause-disrupted adult *H. halys*: *(E)*-2-octenal (Mann-Whitney $U = 3.0$, $n_1 = 3$, $n_2 = 3$, $p = 0.513$), *(E)*-2-decenal ($U = 23.0$, $n_1 = 8$, $n_2 = 6$, $p = 0.897$), tridecane ($U = 22.0$, $n_1 = 8$, $n_2 = 6$, $p = 0.796$). This would suggest that adult *H. halys* produce and store the same amount of these compounds in their glands during diapause and active periods. Response ratios of

these three compounds to the internal standard averaged at 0.32:1. All other compound amounts were therefore estimated assuming a 0.32:1 response ratio to the internal standard.

Table 2.1 Compounds released by agitated diapausing (*n*=8) and agitated diapause-disrupted (*n*=6) adult Halyomorpha halys. 'Compound present' indicates the proportion of bug groups that released the compound. 'Percentage of total' shows the proportion of the compound in relation to the total blend. Amount emitted per bug is given as mean ± *SE*.

	Diapause			Diapause-disrupted		
Compound	Compound present [%]	Percentage of total [%]	Emission [µg] per bug	Compound present [%}	Percentage of total [%]	Emission [µg] per bug
Tridecane	100	53.1	41.7 ± 11.8	100	56.5	43.4 ± 13.6
(E)-2-decenal	100	21.4	18.2 ± 4.2	100	20.3	19.2 ± 5.2
4-oxo-*(E)*-2-hexenal	100	20.6	15.8 ± 6.3*	100	20.3	18.1 ± 5.7*
Dodecane	100	2.6	1.5 ± 0.6*	100	2.0	2.0 ± 0.7*
(E)-2-octenal	37.5	0.2	0.8 ± 0.07	50	0.3	0.7 ± 0.17
(E)-2-decenyl acetate	37.5	0.2	0.06 ± 0.06*	50	0.6	1.0 ± 0.8*
Undecane	12.5	2.0	0.1 ± 0.06*	16.7	<0.1	0.06 ± 0.06*
Tetradecane	n.d.	-	-	16.7	<0.1	0.04 ± 0.04*

**Estimated using ratio of compound to internal standard, assuming a response ratio of 0.32:1;*

n.d. = not detected

2.4 Conclusions

The single compound, tridecane, consistently released by non-agitated diapausing adult *H. halys,* may be detectable, but it is not unique to *H. halys* or pentatomids. Such *n*-alkanes are commonly released biogenic VOCs. Instead, tridecane, *(E)*-2-decenal, 4-oxo-*(E)*-2-hexenal, and dodecane, should be considered collectively for a reliable emissions profile from both diapausing and diapause-disrupted adult *H. halys*.

For the context of chemical detection of *H. halys*, the aforementioned profile is relevant for particular scenario, and has the potential to be applied to large aggregations which have undergone mechanical agitation through transportation, such as the unloading of containers from ships. Were sufficiently sensitive detection methods to be developed, *(E)*-2-decenal could also be considered as an indicator compound for detecting general stink bug populations. In addition, any detection method would also need to overcome the problem of background odours that may potentially interfere with the target volatiles. In terms of practical use, it will be necessary to know how such scenarios as freight and shipping affect diapausing *H. halys* behaviour.

Chapter 3

Halyomorpha halys Group Behavioural Responses to Chemical and Tactile Stimuli

3.1 Introduction

In recent years, New Zealand has introduced fumigation and heat treatment guidelines for cargo considered high risk for presence of *Halyomorpha halys* Stål (Thompson, 2014). Despite these treatments, there have been reported interceptions of the species since the guideline introduction. Currently, visual inspections are the established method for shipping container monitoring. In order to improve this inspection method, or help develop new methods, understanding the behaviour of diapausing *H. halys* aggregations would be beneficial. A sound understanding of *H. halys* aggregation and attraction behaviours has furthered monitoring and management tools in orchards in the USA (Leskey, Khrimian, et al., 2015; Leskey, Wright, Short, & Khrimian, 2012). The identification of large aggregations of diapausing *H. halys* through understanding of behavioural cues has been discussed as a potential means of treating the overwintering bugs within households (Toyama et al., 2011). This potential for detection and treatment of aggregated populations of *H. halys* could similarly be applied to the detection of hitchhiking aggregations located in international freight shipments.

Under the premise of detecting aggregations of *H. halys* by the volatile organic compounds (VOCs) they release, understanding the conditions under which identified VOCs are released could assist in detection plans. Preliminary observations made during work in Chapter 2 suggested that single *H. halys* did not release defensive VOCs when disturbed by mechanical agitation, and groups of three bugs had to be used for volatile collection. The volatile compounds found to make up the defensive odours of *H. halys*, both during active season and when in diapause, are tridecane*, (E)*-2-decenal, 4-oxo-*(E)*-2-hexenal, and dodecane. Whether or not these compounds are released more in group situations should be established, and any group behavioural responses explored. As the literature on behaviour of diapausing *H. halys* is sparse, it is important to confirm cues for both mobility and defensive odour responses for this species. Such knowledge applied to diapausing populations of *H. halys* can assist in

development of improved inspection and detection techniques for border biosecurity. Observations made whilst working with this species indicate defensive responses such as odour release and mobilisation are amplified in aggregatory situations. This study aims to confirm the observation that *H. halys* are more likely to emit defensive compounds when they are in groups, and explore whether this effect can be contributed to intraspecies chemical communication.

3.2 Methods and materials

3.2.1 Field samples of *Halyomorpha halys*

Cohorts of diapausing adult *H. halys* were collected from the field. For this purpose, artificial overwintering sites, i.e., wooden shelters as described by Bergh et al. (2017), were deployed prior to the dispersal of the bugs to potential overwintering sites. Adults from these shelters were then used based on three biological phases. These phases are referred to as (1) autumn dispersal, (2) early diapause, and (3) late diapause, as per below.

The autumn dispersal population were considered to comprise of individuals newly seeking overwintering sites. Shelters for intercepting these were deployed at USDA-ARS Kearneysville, WV (39°21'18.69"N, 77°52'40.71"W) in late September 2016. Subsequently, individual adult *H. halys* were collected from these shelters between 12th and 26th October 2016, when dispersal period was being observed. These bugs were immediately exposed to the relevant experimental conditions. For the early diapause populations, shelters were deployed in Martinsburg, WV (39°24' 50.11"N, 78°01'45.50"W), Keedysville, MD (39°30'18.08"N, 77°44'35.57"W), Keedysville WV (39°29'08.32"N, 77°46'02.04"W), and Shannondale, WV (39°12'28.76"N, 77°47'44.46"W) in September 2016.

For obtaining late diapause populations, shelters were deployed at an organic farm (Redbud Farm) in Inwood, WV (39°23'41.49"N, 78° 4'39.84"W), Mount Weather, VA (39° 3'43.77"N, 77°53'29.65"W), Boonsboro, MD (39°30'20.55"N, 77°44'34.95"W) and Gerrardstown, WV (39°24'22.19"N, 78° 5'54.68"W) in September 2015.

Thereafter, the shelters were collected in early November of each year after diapause onset and maintained in a dark unheated shed at USDA-ARS Kearneysville, WV (39°21'18.69"N, 77°52'40.71"W) under ambient temperature conditions. Adults described as from early or late diapause populations

were inactive, diapausing individuals that remained naturally aggregated. Individuals from both biological phases were taken directly from shelters retrieved from the unheated shed in either November (early populations) or February (late populations) and immediately exposed to the relevant experimental conditions described below.

3.2.2 Effect of group sizing on *Halyomorpha halys'* defensive chemical response

In order to test the hypothesis that increased group sizes lead to increased defensive odour response, groups of 1, 2, 3, 5, and 10 bugs were placed in 36 ml glass tubes and shaken rigorously for one minute. This time length was chosen as preliminary observations had shown that *H. halys* react either within 15-30 seconds of disturbance or not at all. Whether the bugs had released alarm compounds was determined olfactorily by the same one experimenter. This procedure was executed with populations from each biological phase: autumn dispersal, early diapause, and late diapause. To determine whether agitation is a contributing factor to *H. halys* releasing defensive odours, groups of 1, 2, 3, 5, and 10 early diapause bugs were also left stationary for one minute, and release of defensive odours was again olfactorily monitored.

A total of 25 replicates were completed for each group size and biological phase combination. *Halyomorpha halys* defensive response with reference to different group sizes was observed to be similar for the three biological phases (autumn dispersal, early diapause, and late diapause). A statistical comparison between these three phase groups was performed on their distributions of number of bugs with defensive response across the five group sizes, using a Chi-squared test. Then, since no significant difference was found between the groups, the early diapause population was chosen to compare the number of responses within the different group size pairs, with generalised linear models, assuming Poisson distribution through log link function. Comparisons to 0 or 100% response presence groups were made with the Fisher's Exact Tests (FET).

3.2.3 Tactility as a factor for odour release

To test *H. halys* odour responses to different tactile stimuli, single stink bugs were placed in 36 ml glass tubes and exposed to the various stimuli for one minute. Stimuli were both inert and living. Inert

stimulus was in the form of dead *H. halys*, all those used had been soaked in ethanol for six hours and then left to dry overnight, in order to deodourise them. Living stimuli was in the form of *Rhagoletis pomonella* Walsh, commonly called the apple maggot fly; adults of this species were used as they were the largest, non-stink bug, colony insect available, which co-occurs with *H. halys* in the field. As agitation has earlier been shown to be a factor, the treated bugs which had not released odour were then agitated for one minute with the stimuli present. Treatments which single *H. halys* were exposed to were as follows: inert (1 dead *H. halys*), increased inert (10 dead *H. halys*), live (1 living *R. pomonella*), and increased live (10 living *R. pomonella*). Defensive response was identified olfactorily by the experimenter after one minute of stimuli only, and then after one minute of stimuli plus agitation. Twenty five repeats of each treatment were performed, as well as 15 repeats of control bugs undergoing the same conditions without tactile treatments. Fisher's Exact Test was performed to assess significant differences between defensive responses to different tactile treatments.

3.2.4 Exposure of *Halyomorpha halys* to conspecific defensive compounds

Monitoring *Halyomorpha halys* chemical release

To assess whether the odour compounds released by *H. halys* caused other bugs in a population to also release defensive odours, individual *H. halys* from the early diapause population were placed in 36 ml glass tubes and exposed to the individual compounds in turn. Individual standards of tridecane, *(E)*-2-decenal, and dodecane (all >94%, Sigma-Aldrich, Australia), and 4-oxo-*(E)*-2-hexenal (provided by Ashot Khrimian, USDA-ARS, Beltsville, MD, USA, see Chapter 2, Figure 3 for chromatogram and mass spectrum of synthesised compound) were prepared in dichloromethane (DCM). The amount of compound in each treatment corresponded to known emission rates per bug, as shown in Chapter 2 (Table 2.1), and were as follows: tridecane (40 µg), *(E)*-2-decenal (18 µg), 4-oxo-*(E)*-2-hexenal (16 µg), dodecane (2 µg), and clean DCM (2 µL). Compounds were pipetted directly into the glass tube. A single bug was placed in each treated tube and left to stand for one minute. The release of odours could not be measured olfactorily, therefore the tubes were equipped with a portable battery-operated air pump (PAS-500, Spectrex, CA, USA) and headspace was collected through a volatile collection trap

(VCT) containing 30 mg of Super-Q (Analytical Research Systems, FL, USA) at a rate of 400 ml/min for 10 minutes. The VOCs were extracted from VCTs using 250 µl of DCM. A control blank for each treatment was taken using the same apparatus and extraction technique. Samples were stored at -80°C, but kept on dry ice for transport to Lincoln University, New Zealand, where gas chromatography – mass spectrometry (GC-MS) analysis was completed.

GC-MS analysis was performed on a Shimadzu GCMS2010 (Ultra) with an RTX-5MS column (30 m x 0.25 mm I.D.), with GCMSsolutions software. Auto-sampling performed on PALS LHX-xt system.

The GC-MS method used a high pressure 1 µl splitless injection at an injection temperature of 250°C. The GC was operated at a column flow of 0.6 ml/min. The temperature programme started at 40°C for 7 minutes, followed by temperature ramping of 6°C/min until a final temperature of 230°C was reached and held for 5 minutes. The mass spectrometer was run in total ion count mode, with a scanning range 25-550 m/z.

Ten repeats of each treatment were performed, and resulting chromatograms were analysed for the presence of the treatment compound and any additional defensive compounds present. Any chromatograms not containing the treatment compound were discarded. Fisher's Exact Tests (FET) were performed to assess significant differences in compound presence between treatments.

Tracking *Halyomorpha halys* movement

Preliminary observations suggested that groups of early diapause *H. halys* become more mobile upon exposure to defensive odours. To investigate this, the horizontal movement of individual *H. halys* exposed to components of defensive odours was tracked through video recordings. Petri dish arenas (100 x 15 mm) were treated as described below prior to transferring individual bugs into them. Initially the effect of the natural *H. halys* odour was tested; the treatment was prepared by enclosing 10 *H. halys* in the petri dish, mechanically agitating, and removing them upon producing defensive odour. To test the effect of individual components, individual standards of tridecane, *(E)*-2-decenal, and dodecane (all >94%, Sigma-Aldrich, Australia), and 4-oxo-*(E)*-2-hexenal were prepared as above. To test whether the dilution solvent, DCM, had any effect, one set of trials was run whereby the treatment comprised 5 µl clean DCM. The amount of compound in each treatment corresponded to known

emission rates per bugs as above. Compounds were pipetted directly onto the Petri dish. To avoid cross contamination of compound treatments, a single treatment (*n*=10) was run on a day. Controls were also performed every day, which consisted of individual *H. halys* placed in Petri dishes without any additional compounds or agitation (*n*=10). A video visualiser system (R(E)-350, Canon, Inc., Tokyo, Japan) was suspended above five Petri dish arenas with fluorescent backlights and used to track the movement of adults over 1 h. The distance (cm) moved by each bug in the first 10 mins of the trial was tracked using EthoVision software (version 3.1.16, Noldus Information Technology Inc., Leesburg, VA (Noldus, Spink, & Tegelenbosch, 2002)), for full method refer to Morrison et al. (2017c). The room was kept dark and maintained at temperatures between 21 and 24°C for all replicates, with R.H. >40%. A total of 120 adults were tested during the experiment.

For all treatments, a total of 10 treatment and 10 control replicates were performed. To assess if any treatments were significantly different from their controls, 2-sample t-tests were performed for each treatment and control set. Possible outliers were identified using distribution box plots and Grubb's outlier test and were subsequently removed from the data set. The data set for the 4-oxo-*(E)*-2-hexenal treatment followed a non-normal distribution, so both control and treatment data sets were transformed by the addition of 0.05 (to correct for 0 values in control set) and square root function. Only the treatments that were found to have a significant effect on bug locomotion, 4-oxo-*(E)*-2-hexenal and dodecane, were carried forward for further analyses. To assess the effect of running trials on different days, the control sets paired with significant treatments were tested for using a 2-sample t-test. There was no significant day effect identified, therefore a 2-samples t-test was performed on the 4-oxo-*(E)*-2-hexenal and dodecane treatment data sets to establish whether one compound had a higher effect on the distance moved than the other. Both data sets were square root transformed prior to testing to normalise the distributions.

3.3 Results

For access to chromatographic data and full EthoVision data see Appendix C.

3.3.1 Effect of biological state and group sizing on *Halyomorpha halys* defensive chemical release

There was a positive relationship between the number of *H. halys* in a group and the number of defensive response events across all biological states tested (Figure 3.1A). Notably there were no responses from the stationary control group regardless of group size. A Chi-Square test showed that the biological states tested had no significant effects on the number of defensive response events (X^2 = 0.661, *df* = 6, *p* = 0.995). As no difference was identified between the states, the early diapause data was taken forward for further analysis and the early diapause populations were used for following experiments, assuming similar behavioural patterns. Significance indicators from results of generalised linear model and Fisher's exact tests performed on early diapause data: 1 – 2: (FET, $p< 0.001$), 2 – 3 (GLM, $p= 0.251$), 3 – 5 (GLM, $p= 0.192$), 5 – 10 (FET, $p= 0.110$), 1 – 10 (FET, $p< 0.001$). Results reveal a significant difference in release responses, showing that the more *H. halys* present in a group, the more likely that group is to release defensive odours.

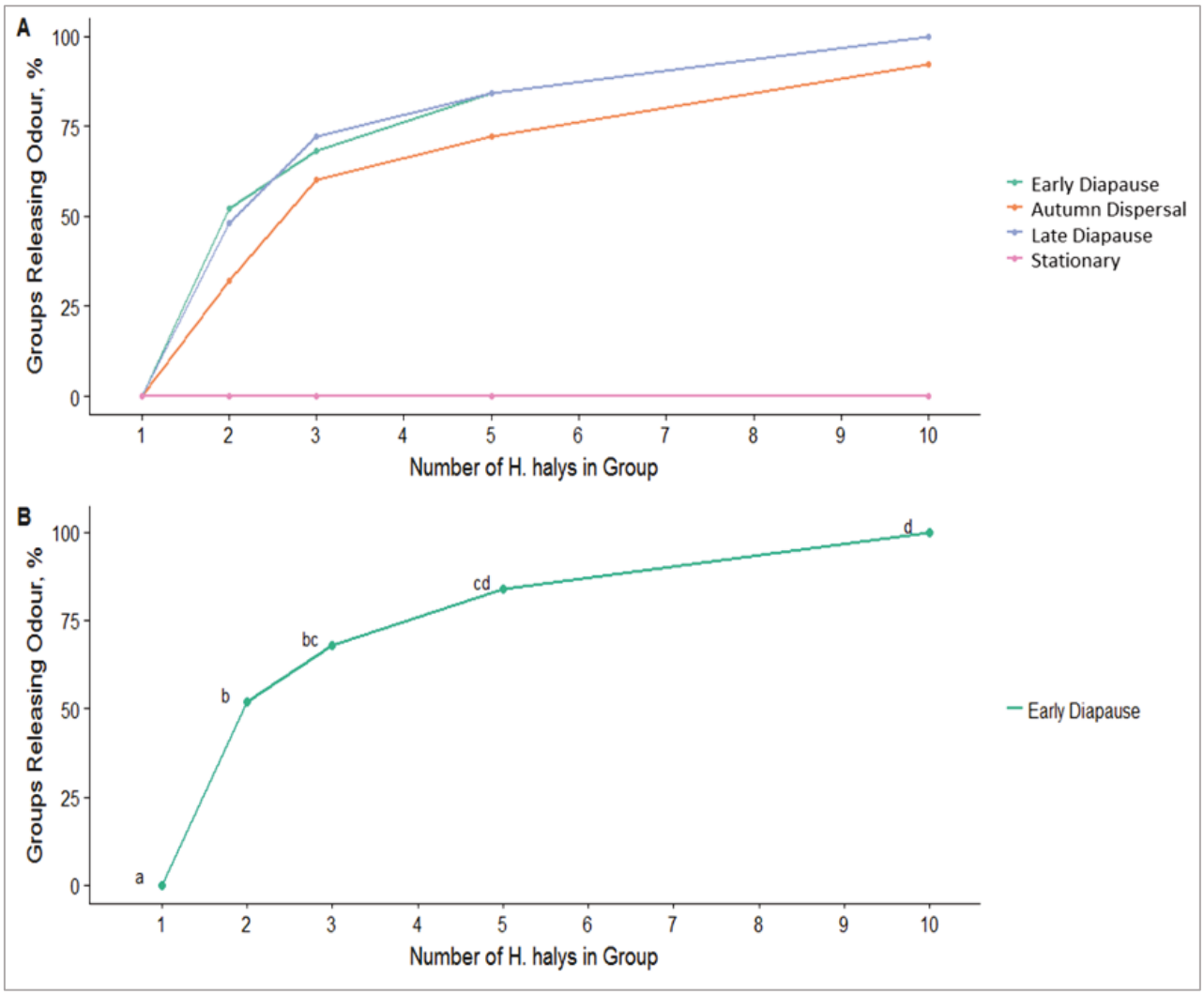

Figure 3.1 **A) Line graph showing the odour release responses [%] of groups of 1, 2, 3, 5, and 10 *Halyomorpha halys* when exposed to mechanical agitation, in three biological states, with stationary control group data shown. B) Line graph showing the defensive release responses of groups of 1, 2, 3, 5, and 10 *H. halys,* from early diapause population only, when exposed to mechanical agitation. Different letters above data points indicate statistically significant differences ($p < 0.05$).**

3.3.2 Effect of tactile stimuli on *Halyomorpha halys'* defensive chemical release

As presented in Table 3.1, the outcome of the tactility experiment shows that the treatments worked synergistically. Stimuli-only treatments had no statistically significant effect from the control samples, and in the single live and 10 live treatments, the stimuli-only was significantly lower than stimuli +

agitation treatment responses (FET, p= 0.023 and p<0.001, respectively), showing that mechanical agitation is a key condition for defensive chemical release in this scenario.

The responses recorded for the 10 inert, stimuli + agitation treatment were neither significantly different from the control, stimuli + agitation treatment nor single inert, stimuli + agitation treatment (FET, p= 0.490). The responses recorded for single live, stimuli + agitation treatment were somewhat higher than those recorded for 10 inert, stimuli + agitation treatment (FET, p= 0.074), and significantly higher than control, stimuli + agitation (FET, p= 0.004). Responses recorded for 10 live, stimuli + agitation treatment were significantly higher than single live, stimuli + agitation (FET, p= 0.020). These differences among stimuli + agitation treatments show that stimulus is also an important factor of defensive chemical release, with live stimuli having a greater effect than inert stimuli.

Table 3.1 Counts showing number of defensive compound release events from individual *Halyomorpha halys* exposed to tactility treatments (*n*= stimuli *n*, stimuli + agitation *n*). Superscript letters indicate statistical groupings. Control *H. halys* are not exposed to any stimuli. Inert stimuli were in the form of dead, deodourised *Halyomorpha halys*. Living stimuli were live apple maggot flies (*Rhagoletis pomonella*).

Treatment	Positive Olfactoral Response	
	Stimuli Only	Stimuli + Agitation
Control *(n=25)*	0^{a}	0^{a}
Single Inert *(n=25)*	0^{a}	0^{a}
10 Inert *(n=25)*	0^{a}	2^{ab}
Single Live *(n=25, 24)*	1^{a}	7^{b}
10 Live *(n=25, 23)*	2^{a}	15^{c}

3.3.3 Effect of exposure to conspecific defensive compounds on *Halyomorpha halys* individual compound release

The effect of exposure to conspecific defensive chemicals shows that the contributing factor here would be the single compound 4-oxo-*(E)*-2-hexenal. As the compound tridecane appeared as a contaminant in most system blank controls, this was removed from results analysis. The 4-oxo-*(E)*-2-hexenal treatment was the only compound to trigger a significant increase in release of the marker compounds, *(E)*-2- decenal and dodecane, in comparison to the control treatment (FET, p=0.002 and p<0.001, respectively).

Table 3.2 **Effect of exposure to conspecific defensive compounds on *Halyomorpha halys* individual compound release, showing numbers of events of defensive responses presence. Control groups were exposed to DCM solvent. * signifies compound present in system blank for treatment set.**

Treatment	**Compound Detected**			
	Tridecane	*(E)*-2-Decenal	4-Oxo-*(E)*-2-Hexenal	Dodecane
Control *(n=10)*	10*	1	0	1
Tridecane *(n=10)*	-	0	0	0
(E)-2-Decenal *(n=8)*	8*	-	0	0
4-Oxo-*(E)*-2-Hexenal *(n=7)*	7	5	-	7
Dodecane *(n=9)*	9*	0	0	-

3.3.4 Effect of exposure to conspecific defensive compounds on *Halyomorpha halys* individual movement

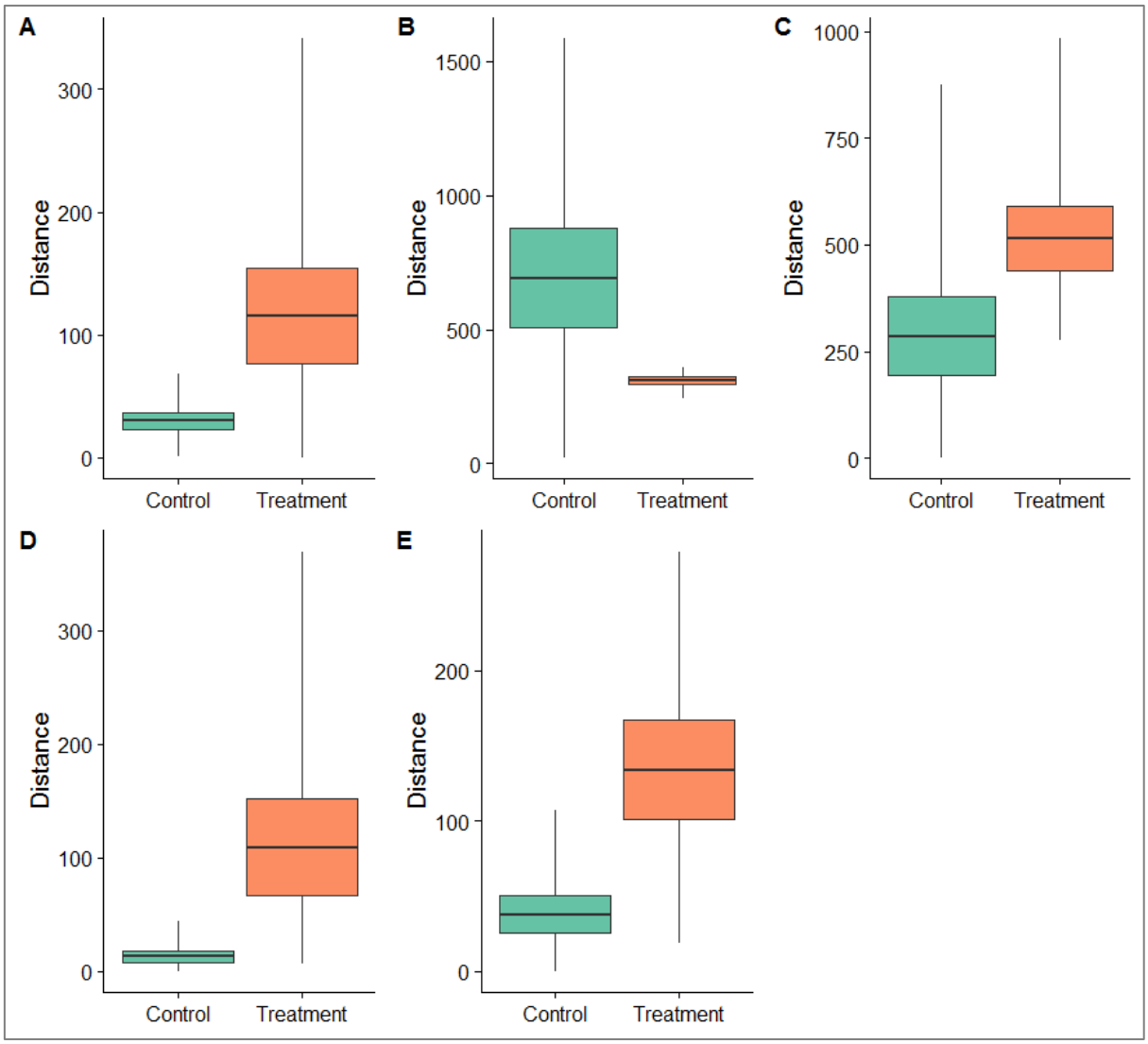

Figure 3.2 **Effect of conspecific odour compounds on the locomotory response of *Halyomorpha halys.* Box plots show means of each data set ± *SEM*, tails extreme values. Ten repeats of every treatment and control set. A) Odour produced by 10 *H. halys* treatment and respective control set (2-sample t (9) = -2.16, *p* = 0.059). B) Tridecane treatment and respective control set (2-sample t (9) = 2.04, *p* = 0.071). C) *(E)*-2-decenal treatment and respective control set (2-sample t (17) = -1.92, *p* = 0.071). D) 4-oxo-*(E)*-2-hexenal treatment and respective control set (2-sample t (12) = -2.80, *p* = 0.016). E) Dodecane treatment and respective control set (2-sample t (10) = -2.75, *p* = 0.020).**

The control treatment of clean DCM had no significant effect on *H. halys* mobile against controls (2-sample t (16) = -0.61, p = 0.548). The 2-sample t-test showed no significance (p= 0.059) supporting that the whole odour treatment caused *H. halys* to travel larger distances than the control set. From this, the two single compound treatments which had a significant effect on the treated *H. halys* in comparison to their control counterparts were 4-oxo-*(E)*-2-hexenal (p= 0.016) and dodecane (p= 0.020); both treatments increased the distance walked by individual *H. halys*. The two respective control data sets for these treatments did not differ significantly (2-sample t (12) = -1.82, p= 0.094), therefore the two treatment data sets were compared directly. No significant difference was found between the two treatments (2-sample t (16) = -0.85, p= 0.409).

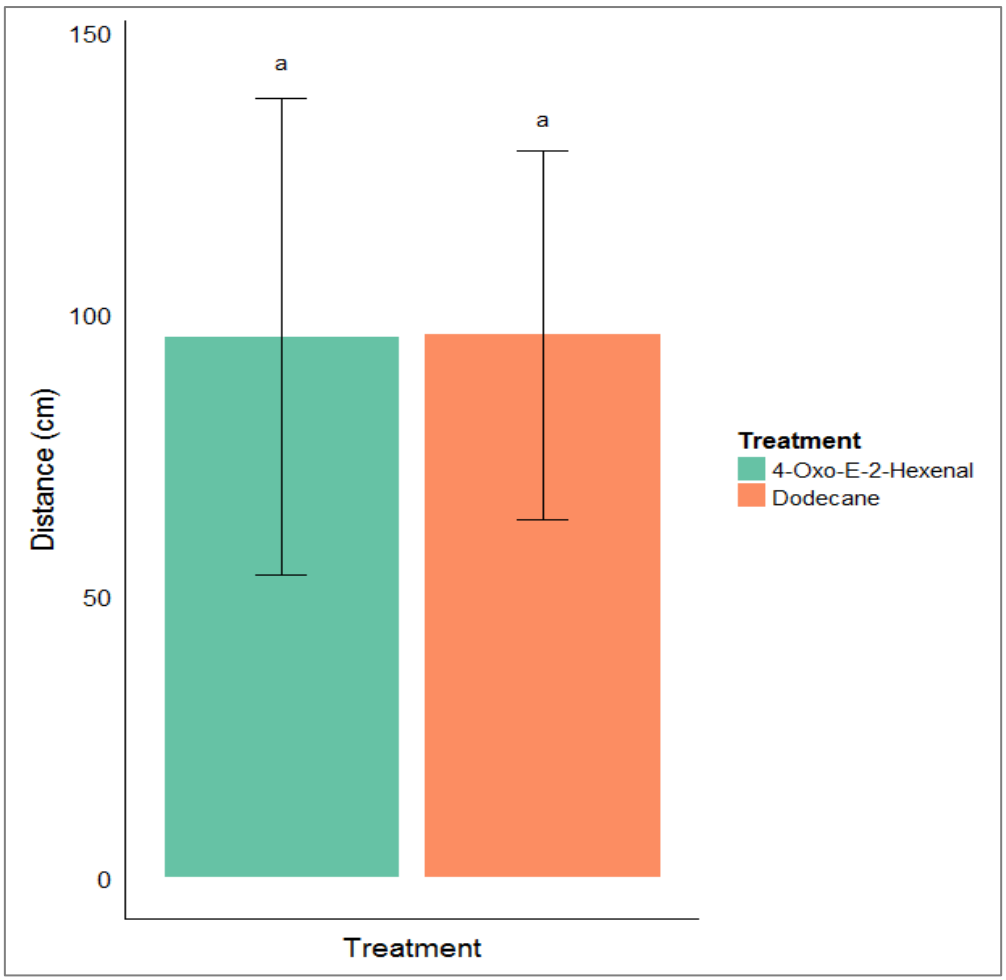

Figure 3.3 **Mean ± *SEM* to show comparison of distances (cm) moved by individual *Halyomorpha halys* when exposed to single compound treatments, 4-oxo-*(E)*-2-hexenal and dodecane, (2-sample t (16) = -0.85, p= 0.409).**

3.4 Discussion

3.4.1 Effect of biological state on *Halyomorpha halys'* release of defensive odours

In testing the effects of group size and biological state on defensive odour release, there was no significant difference in defensive odour responses between the three biological states tested. These states are all overwintering related: autumn dispersal are searching for overwintering sites, and both other states (late and early), the bugs remained in diapause. Conversely, in Chapter 2, it was established that broader biological states of *H. halys* investigated did affect the bug's propensity for releasing defensive VOCs. These states were when (i) *H. halys* is in diapause, and (ii) after *H. halys* had been artificially removed from diapause through exposure to warm temperatures, long photoperiods, and provision of food sources. The *H. halys* population in diapause in Chapter 2 would be classed as the late diapause population here, and was found to be significantly more likely to release defensive VOCs than the diapause-disrupted population (see 2.3.2). Reactions of pentatomids to mechanical cues can generally range from moving away from the threat to releasing their defensive odours (Krall et al., 1999). Since the purpose of *H. halys* diapause is to maintain a slow metabolism and uphold an appropriately secure overwintering location, releasing defensive odours would be more apposite for *H. halys* in diapause related states than relocating.

3.4.2 Effect of group size and stimuli on *Halyomorpha halys'* release of defensive odours

There was a positive relationship between the number of *H. halys* in a mechanically agitated group and the number of defensive response events; this is exhibited in all three biological states tested. The increase in likelihood of release with group number was not linear, i.e. it was more likely for groups of ten to release than individual bugs, but not 10 x more likely. So, this does appear to be a group or aggregated bug effect, which could be triggered by one group member or a few of the group members, creating an amplification effect. Lack of any odour responses from the stationary control groups show that the high mechanical agitation plays a major role to trigger defensive responses under these conditions. This also establishes that simple aggregation/grouping of *H. halys* is not the controlling factor for defensive odour release.The mechanisms used by active season pentatomid species are to

aggregate as visual defence against predators, for mating, and to facilitate easier plant feeding (Alcock, 1971; Eisner & Kafatos, 1962). Defensive aggregation of non-aposematic insects like *H. halys*, is used by individuals to decrease the likelihood of being attacked by predators, or if a predator attacks one member, they would be repelled by the bad taste and be less likely to revisit that aggregation (Alcock, 1971). An increase in chemical defence signals against predators would also benefit aggregated groups of pentatomids. Aggregative behaviour likely acts both defensively, and as a means of sharing an appropriate overwintering location (Toyama et al., 2006). As *H. halys* aggregate to overwinter, settled grouping should not provoke a defensive reaction. However, were an overwintering population to be physically agitated, then the release of defensive chemicals could protect an aggregation from potential predation or disturbance of an overwintering site.

Before this study, there had been little research on *H. halys* threat recognition or response to stimuli during their diapause season. One study established that larger sized web spiders within anthropogenic structures are successful at trapping and consuming *H. halys* during the overwintering period (Morrison, Bryant, Poling, Quinn, & Leskey, 2017b), and there are reports of birds and small mammals consuming the bugs in their immobile state. However, which threat types trigger *H. halys* to either disperse or release defensive odour has not been firmly established. When considering the diapause state, it would intuitive to assume that dispersal from a safe overwintering location would not be a viable option. Krall et al. (1999) investigated tactile threat responses in the pentatomid species *Cosmopepla bimaculata* Thomas during its active season. Adult *C. bimaculata* did not emit defensive odours when prodded with a wooden dowel; it simply moved away from it, sometimes emitted odours when picked up and roughly handled, and always emitted when placed in the experimenter's mouth and pressed between tongue and palate. This is somewhat in agreement with the tactility experiment results in this study: inert stimuli had no effect. Here we have shown with *H. hays* that increased amounts of living stimuli which can trigger stink bugs to emit defensive odours. This would also show that pentatomid species can perceive when a threat is escapable, and therefore not immediately trigger defensive compound release. The species used as stimuli in this study, *R. pomonella,* was chosen purposefully as a non-predatory, live stimulus for *H. halys*. This was to assess whether the

defensive response would be purely to tactile stimulation, rather than as a predatory response. The tactility does indeed contribute to the response. Alcock (1971) observed intra-species anti-social behaviour in formed non-reproductive contact groups of the pentatomid *Euschistus conspersus* Uhler, recording actions of aggressive intent within a wild aggregation. The main reason for anti-predator aggregations is for aposematic insects to increase their warning colour signal to predators. For non-aposematic species, such as *E. conspersus* and *H. halys*, the aggregations may then facilitate easier plant feeding. It is these aggregations that were observed presenting intra-species aggression, so it is possible that threats can be posed by non-predatory species, and even conspecifics. As shown by both the tactility experiment (Table 3.1) and the control groups shown in Figure 3.1, a significant increase in defensive chemical release is only elicited when mechanical agitation is added as a condition. As discussed, perhaps it is the agitation which makes the condition seem inescapable, and more threatening.

3.4.3 Effect of exposure to conspecific defensive compounds on *Halyomorpha halys'*

The significant effects of 4-oxo-*(E)*-2-hexenal and dodecane on the distance travelled by individual *H. halys* would suggest that these compounds play equal roles as an alarm pheromone, since both caused an increase in movement. Surprisingly both individual components had a larger behaviour modifying effect than the combined "natural odour", which showed no statistically significant effect ($p = 0.059$). As the clean DCM solvent treatment showed no significant differences in distance moved, all differences observed were therefore assumed to be true effects. It has previously been suggested that tridecane could act as a mediation chemical to regulate pheromonally-induced behaviours in pentatomids (Harris et al., 2015; Zhong et al., 2017), and in some circumstances tridecane was found to work synergistically with *(E)*-2-decenal as a predator repellent (Eliyahu, Ceballos, Saeidi, & Becerra, 2012). Therefore, the "natural odour" treatment may have a lesser effect as the active components are diluted/mediated by the tridecane, as the most abundant compound. 4-oxo-*(E)*-2-hexenal was not found to have a repellent effect on the predatory Chinese praying mantid (*Tenodera aridifolia sinensis* Saussure) (Noge, Prudic, & Becerra, 2012). Although this compound has been shown as an effective

deterrent against predatory ant species (Eliyahu et al., 2012). Perhaps, the compound 4-oxo-*(E)*-2-hexenal, is released not only as a defensive compound, but as an alarm pheromone, signalling to other *H. halys* in the population to either disperse or release their own defensive compounds. Therefore 4-oxo-*(E)*-2-hexenal is likely to be a multi-functional semiochemical, acting as both a pheromone and allomone, like many semiochemicals released by Heteroptera species. *(E)*-2-alkenals isolated from Heteroptera have been shown to repel generalist predators (Noge et al., 2012). *(E)*-2-decenal specifically has been repeatedly confirmed as an allomone repellent against both insect and non-insect predators of heteropterans, both individually and synergistically (Eliyahu et al., 2012; Gregorovičová & Černíková, 2015; Krall et al., 1999; Waterhouse, Forss, & Hackman, 1961; Zhong et al., 2017). Zhong et al. (2017) investigated inter- and intra-species roles of semiochemicals released by *H. halys* within a *H. halys* and *Trissolcus japonicus* Ashmead host-parasitoid system. The kairomonal function of the two most abundant compounds of *H. halys* defence chemicals, *n*-tridecane and *(E)*-2-decenal, was explored and discussed; the former was found to attract the parasitoid and the latter repel.

Intra-species Y-tube tests of *n*-tridecane and *(E)*-2-decenal revealed that *n*-tridecane was an attractant to male *H. halys* and *(E)*-2-decenal was a repellent to all adult *H. halys*. Results described there would suggest that *(E)*-2-decenal could act as an alarm pheromone for *H. halys* in high concentrations, but that this biological function would need further investigation (Zhong et al., 2017). The only concentration tested in the present study was approximate to the amount of compound released by a single *H. halys*, therefore the lack of response shown here may be from the low concentration. A weak increase in distance travelled was stimulated by *(E)*-2-decenal compared to its control set, this may be showing a similar alarm effect. It is well established that pentatomid species release defence compounds which often serve the dual purpose of acting as an alarm pheromone (Aldrich, 1988; Kou, Tang, & Chow, 1989). *(E)*-2-hexenal has been identified as both a defensive chemical and a pheromone which causes intra-species alarm and dispersal (Ishiwatari, 1974). This effect was observed in first to third instar nymphs of *Eurydema rugosa* Motschulsky, *Nezara viridula* Linnaeus, and *Eurydema pulchra* Westwood. *Eurydema rugosa* nymphal odour components act as both an attractant and alarm pheromone, depending upon concentrations released (Ishiwatari, 1976). Todd (1989) reported similar

effects with *N. viridula* nymphs, which produce tridecane in small amounts as an attractant and large amounts as a dispersal signal. Early instar nymphs and adult heteropterans generally secrete different odour compounds, utilising different defence and alarm tactics from one another (Borges & Aldrich, 1992). This is down to early instar's low mobility, whereby they need stronger and longer lasting chemical protection.

3.5 Conclusion

The likelihood of diapausing *H. halys* releasing their signature defensive odours is increased when there are increased numbers of the bugs present. This response can be associated with a number of factors. The most significant of these are high mechanical agitation which plays a major physical role, and live tactility also contributes. Whether these effects are observed under realistic conditions needs to be assessed. Considering a biosecurity context, it would be prudent to understand these effects when aggregations of *H. halys* are exposed to more shipping-like movements.

This study shows that the defensive compounds released by *H. halys* act as an alarm pheromone as well as a defensive allomone. When exposed to dodecane and 4-oxo-*(E)*-2-hexenal, *H. halys* are triggered to disperse, and when exposed to the latter, the bug will release its own defensive odour. Tridecane appears to work as a mediation chemical, diluting the effect of both the active components on the distance moved by a single bug. For diapausing *H. halys*, this could be a benefit to prevent relocation, and induce defensive odours instead.

Chapter 4

Brown Marmorated Stink Bug: A Simulated Voyage

4.1 Introduction

As of November 2017, *Halyomorpha halys* Stål has successfully invaded and established in 44 states in the USA, Canada, and nine European countries (Italy, France, Hungary, Switzerland, Germany, Liechtenstein, Greece, Serbia, and Romania), with recent incursions reported in Russia, Georgia, and Bulgaria (Gapon, 2016; Haye et al., 2015; Milonas & Partsinevelos, 2014; Simov, 2016; Valentin et al., 2017; Vetek et al., 2014). Initial invasions of each region can be attributed to human assisted movement (Valentin et al., 2017). It has now been established that, genetically, the Eastern USA populations of *H. halys* all originated from one incursion of mated females from China (Faúndez & Rider, 2017; Xu et al., 2014). After establishing in every Northern Hemisphere continent, the species has now been reported in Chile, its first Southern Hemisphere invasion (Faúndez & Rider, 2017). This being the case, biosecurity in non-invaded, climatically compatible countries, such as New Zealand, must be sufficient to prevent entry of establishing populations. As discussed in Chapter 1, *H. halys* form large aggregations to overwinter, and it is their propensity for aggregating in small, "safe" spaces that result in these aggregations hitchhiking unnoticed during Northern Hemisphere winter months (October – March). Interceptions of *H. halys* at the New Zealand border dramatically increase during these months, and the highest proportion of bugs found are attributed to ship containers and related cargo (Cath Duthie, MPI, personal communication). Figure 4.1 shows a popular shipping route; cargo ships dock at many ports in Europe and the USA before crossing the Pacific to New Zealand and then Australia, creating an ideal pathway for hitchhiking *H. halys*. Understanding of diapausing *H. halys* behaviour during and post a trans-Pacific voyage may lead to improved risk assessment and pathway risk management.

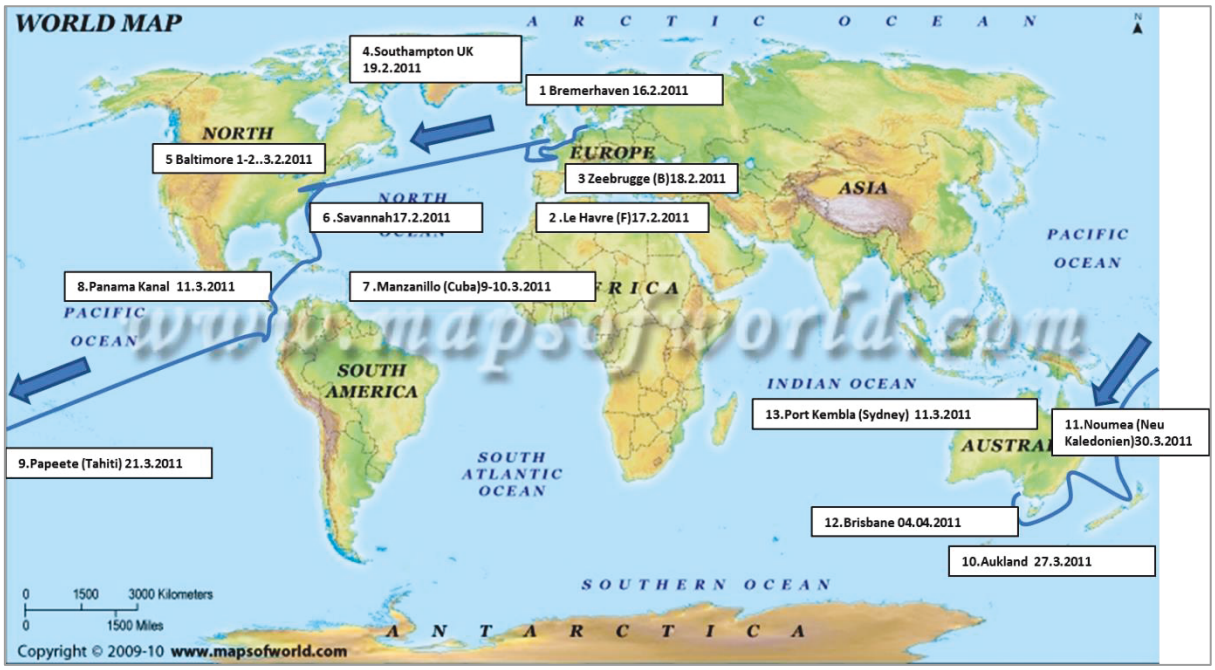

Figure 4.1 World map showing the popular shipping route undertaken by Tamerlane, a Wallenius Wilhelmsen Logistics cargo ship. Map provided by Niklas Blomqvist (Wallenius Wilhelmsen Logistics, personal communication).

In previous chapters, the emission of defence compounds and group interactions by diapausing *H. halys* have been characterised. Chapters 2 and 3 included volatile collection experiments which required the experimenter to approximately replicate the movement effects of shipping on the bugs (see 2.2.3, 3.2.2, and 3.2.3). This was achieved via high mechanical agitation. This was not intended to be a realistic simulation of what *H. halys* would experience within shipping situations, but was sufficient for the collection and characterisation of the volatile profile of agitated *H. halys*. Once this profile was established, the four compounds have been used as indicators of agitation. Findings in Chapter 3 suggested that the tactility of an aggregation combined with agitation elicits a defensive response from *H. halys*. It therefore follows that the varied motion associated with shipping could cause diapausing *H. halys* to release defensive odours. In the same chapter it was established that exposure to conspecific defence compounds, specifically the 4-oxo-*(E)*-2-hexenal component, causes diapausing *H. halys* to move further and/or elicit release of defence compounds. Thus, if shipping-type movement elicits release of defensive odours within an aggregation, this could amplify into a stronger

defence compound signal or induce bugs to become mobile. As previously suggested, *H. halys* volatiles could be a means of detecting the species in cargo, therefore, a simulation of realistic shipping movement and the effect this has on *H. halys* volatile organic compound (VOC) emissions would be a valuable means for predicting the success of such a premise.

Bug mobility is an important risk factor to consider when focussing on possible border inspection and detection procedures. As mentioned, mobility of aggregations could be affected by the movement of a ship and release of conspecific defence compounds. Another variable introduced by shipping that could have an effect on mobility is the drastic temperature changes experienced by cargo ships travelling from the Northern to the Southern Hemisphere. During the target months (October – March), aggregations of *H. halys* hitchhiking within containers would experience an increase in temperatures, which is a factor known to contribute to terminating their diapause state (Kiritani, 2007). The following experiments simulated shipping motion and temperature changes within a container in order to determine their effects on the odour release and behaviour of aggregated, diapausing *H. halys* populations.

4.2 Methods and materials

4.2.1 Populations of *Halyomorpha halys*

Adult *H. halys* used for this study were taken from the early diapause cohorts described in Chapter 3 (see 3.2.1) and immediately exposed to relevant experimental conditions.

4.2.2 Simulation of shipping container movement

Population resettling

Metal shelters described in Chapter 2 (see 2.2.2) were mimicked and scaled down to 1/3rd original size (6 × 7.5 × 7 cm, H × L × W). The scale down was necessary to not overload the robot, which advises not to exceed 7 kg for maximum movement. Populations of *H. halys* were removed from the diapausing cohorts and resettled into metal shelters, as per resettling procedure described in Chapter 2 (see 2.2.2). Each metal shelter contained 34 resettled adult *H. halys* (2.4M: 1F, this ratio was taken from

preliminary observations of wild *H. halys* settling into overwintering shelters). This procedure causes minimal disruption to the bugs, and has no lasting effect on diapause.

Setup

Four metal shelters containing resettled populations were attached to the robot arm at a time. Simultaneously, four metal shelters containing resettled populations were placed in the same room on a table, to remain stationary and therefore act as control populations against those undergoing movement. All shelters were individually wrapped in oven roasting bags in order to hold any released volatiles in the headspace, then wrapped in tin foil to keep populations in the dark and at stable temperatures. The room was maintained at 19.15 ± 0.04°C.

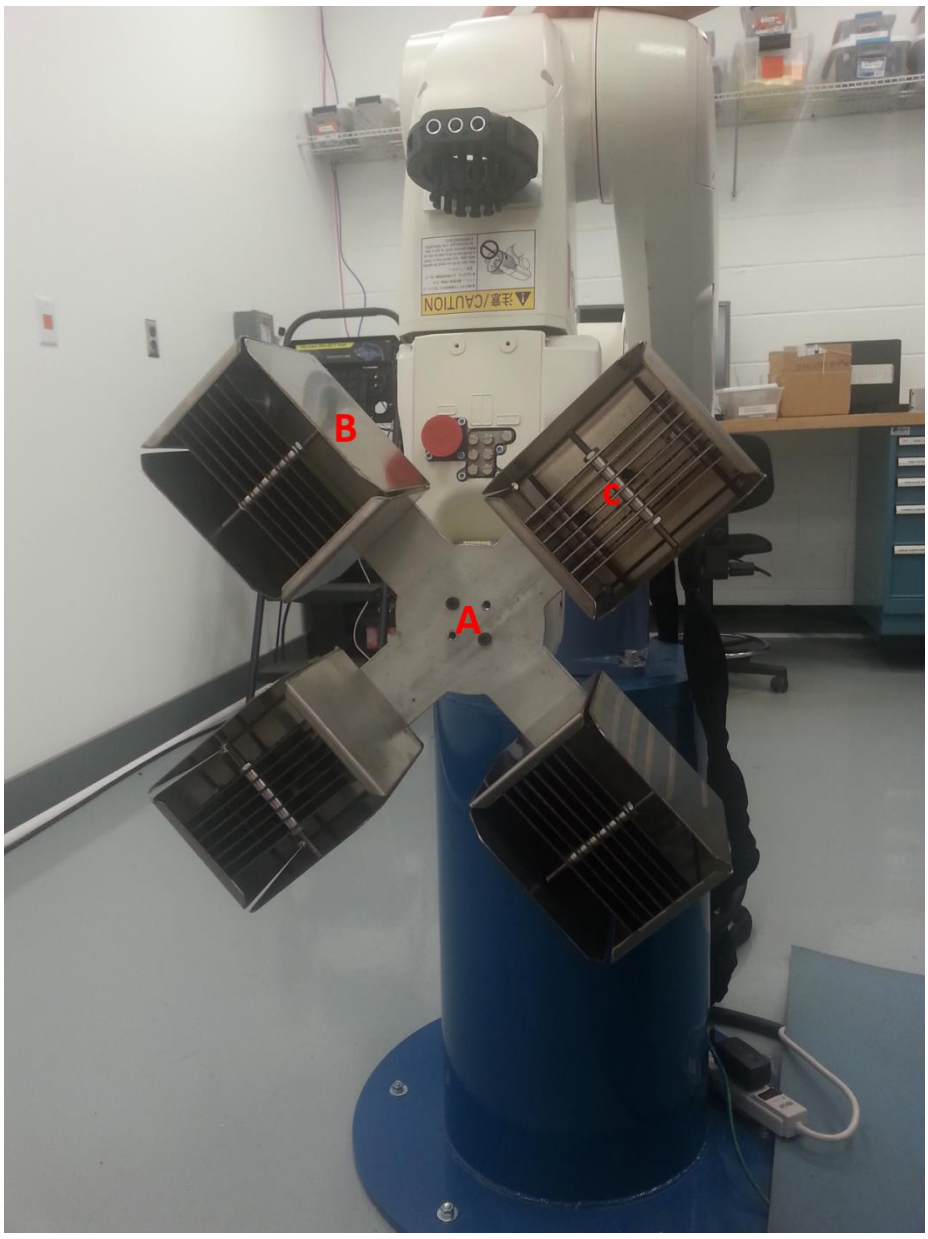

Figure 4.2 **Experimental setup showing a 6-axis VS-6577G-B Denso Robot with 934 mm reach, with attached metal shelters containing diapausing *Halyomorpha halys* colonies. A) Metal cross structure B) Metal shelters (6 × 7.5 × 7 cm, H × L × W) C) Inserts constructed from 7 ~2 mm wide metal sheets (7.5 × 6.5 cm, L × W) spaced 90 mm apart, affixed in place with three screws running the length of the box and a series of nuts.**

Movement Simulation Programme for Denso Robot

The 6-axis VS-6577G-B Denso Robot was programmed to perform two actions; rocking, which was to simulate ship movement, and dropping, which was to simulate the dropping of containers during loading and unloading. In the rocking movement, the robot was programmed to traverse the lower ¼

of a circle's circumference, where the circle's radius was 425 mm. The speed of the robot for each rock cycle of back and forth had a Gaussian distribution to simulate the motion of waves. The drop movement program consists of instructions to slowly raise the robot's end effector and then at maximum speed, move quickly downwards and stop without any deceleration. This is not a free-fall situation, because the robot's movements are controlled by motors. The trajectory of the drop motion is straight down to the ground. The distance of the drop motion was 637.5 mm. This programming was based on previous conducted to model the movement of marine vessels (Perez & Fossen, 2007).

VOC Sampling and Observations

The four metal shelters with *H. halys* populations remained on the robot simulator for 5 days. Each bug population was sequentially sampled as follows: time point T1 (day 1, after simulated ship container loading), T2 (day 1, 2 hours after simulated wave motion), T3 (day 5, immediately preceding simulated unloading), T4 (day 5, immediately following simulated unloading), T5 (day 5, 2 hours after immobilisation).

At each sampling time point, volatiles were collected from the headspace of each bug population through a volatile collection trap (VCT) containing 30 mg of Super-Q (Analytical Research Systems, FL, USA) at a rate of 400 ml/min for 10 minutes. The VOCs were extracted from the VCTs using 250 µl of DCM with 1.5 ng/µl tetralin (Sigma-Aldrich, Australia) as an internal standard. Samples were stored on dry ice and analysed at Lincoln University, New Zealand. GC-MS analysis of volatiles was performed as described in Chapter 3 (see 3.2.4).

At all five time points, the number of mobile *H. halys* in each population was also counted, and, immediately after T5, the number of dead bugs was counted. Counts of mobile *H. halys* were conducted using a red light source, in order to not introduce white light to the bugs, as populations were kept in the dark at all times to eliminate light as a variable.

Statistical analysis of movement simulation data

The movement simulation experiment was repeated three times with four new bug populations (n=12), with twelve accompanying stationary control shelters. Resulting chromatograms were analysed for the presence of the four *H. halys* alarm compounds identified in Chapter 2: tridecane, *(E)*-

2-decenal, 4-oxo-*(E)*-2-hexenal, and dodecane. Fisher's Exact Tests were performed to assess significant differences in compound presence, bug mobility, and mortality rates between the treatment and control shelters.

4.2.3 Simulation of shipping journey temperatures

Population resettling

Populations of *H. halys* were removed from the diapausing cohorts and resettled into wooden shelters described in Chapter 2 (see 2.2.1) (Bergh et al., 2017), as per resettling procedure described in Chapter 2 (see 2.2.2). Each wooden shelter contained 68 resettled adult *H. halys* (2.4M: 1F). This procedure causes minimal disruption to the bugs, and has no lasting effect on diapause. All diapausing populations were resettled into 24 wooden shelters. Each wooden shelter was secured in its own mesh rearing cage for the course of the experiment to allow observation of the bugs whilst keeping them contained.

Temperature Simulation and Setup

To simulate the temperature changes experienced when travelling, eight wooden shelters were placed in a dark temperature controlled chambers. The temperature of the chamber was changed as follows: started at 12°C, slowly increased to 30°C over 7 days, held for 12 days, and slowly decreased to 23°C over 7 days (Figure 4.4). The temperatures are taken from data provided by Niklas Blomqvist from Wallenius Wilhelmsen Logistics (Figure 4.3). Eight shelters were placed in a temperature stable refrigerator (5.16±0.17°C), and eight shelters were placed back into the dark, unheated shed at USDA-ARS Kearneysville, WV, kept at ambient temperatures (10.56 ± 0.17°C). These were considered as control treatments for comparison. All treatment groups remained in described conditions for 26 days (02/11/2016 – 28/11/2016), and all were kept dark for that period.

Mobility and Mortality Counts

Every two days during the 26-day period, the number of mobile *H. halys* in all treatment and control populations were counted using a red light source. In order to avoid disturbing the diapausing populations, mobile bugs were classed as bugs which were outside of the wooden shelter within the mesh cage and were observed as clearly moving around; for instance some bugs settled in the corners

of the mesh cages and did not move for the entirety of the experiment, these were not classed as mobile. The mesh cages were not opened and the shelters were not touched or moved for the entire 26-day period.

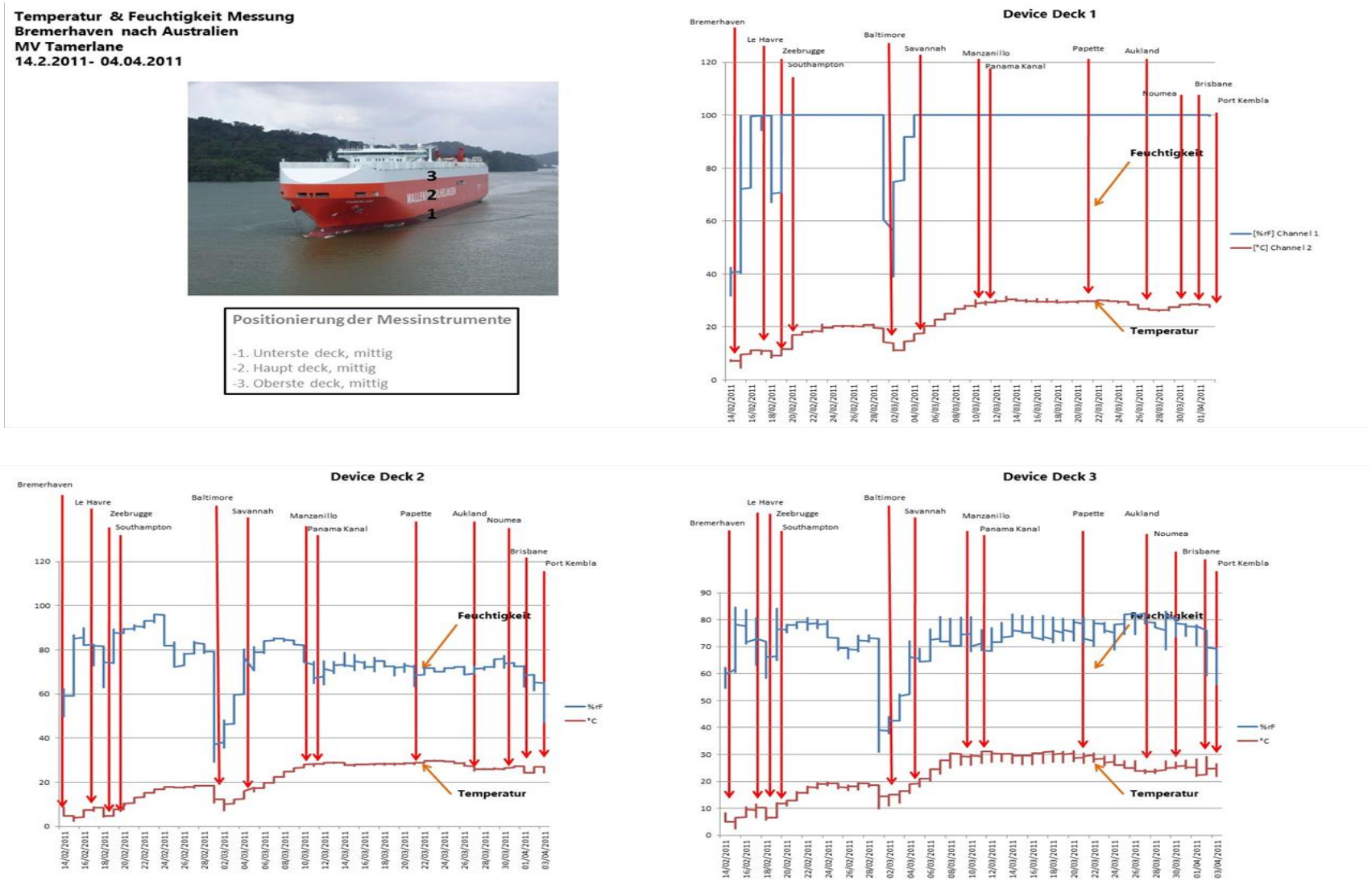

Figure 4.3 Data provided by Niklas Blomqvist from Wallenius Wilhelmsen Logistics showing the ship, Tamerlane, positions of data loggers carried, and full results from data loggers on each deck.

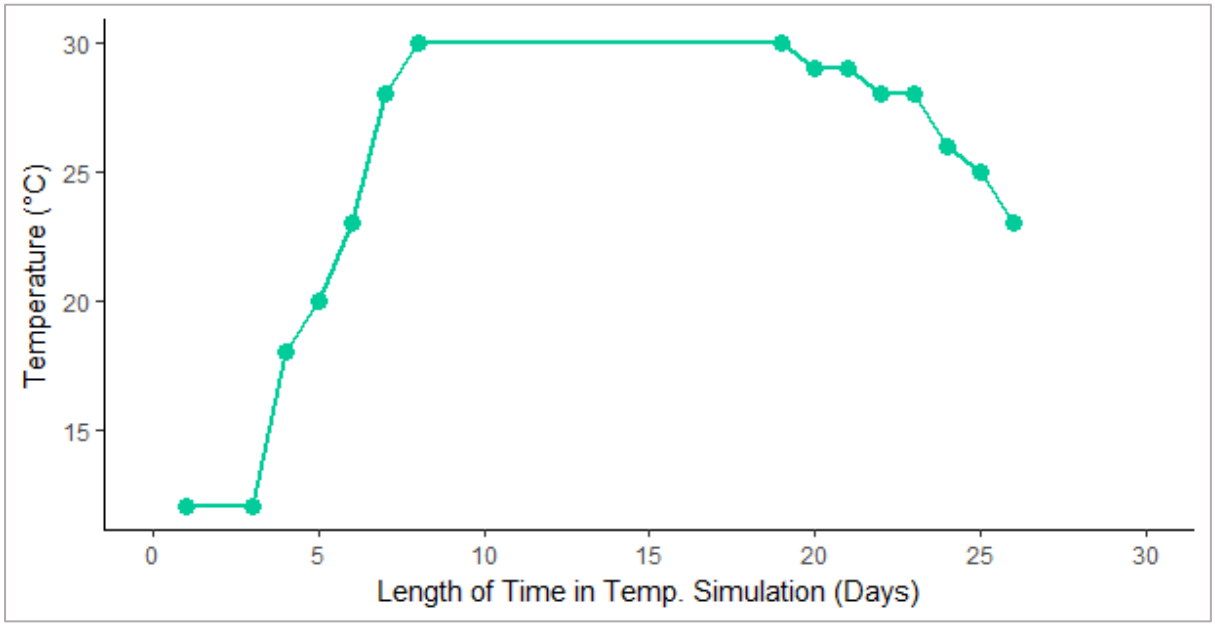

Figure 4.4 Average temperatures extracted from Tamerlane data, showing the temperature fluctuation simulation followed in this 26 day study.

Dissection of post-experiment females

Female *H. halys* found mobile at the end of the temperature simulation treatment experiment were removed and freeze-killed overnight. Three females were also removed from the naturally diapausing cohorts which had not undergone any experimental treatments and freeze-killed. All frozen samples were dissected in order to assess stage of reproductivity, and identify whether mobility due to temperature increases correlated to coming out of reproductive diapause. Samples were thawed for at least 20 minutes prior to dissection, and covered in Ringer's solution (1 l distilled water, 9.1 g/l NaCl, 0.52 g/l KCl, 0.2 g/l $CaCl_2$, 0.8 g/l $MgCl_2$). Legs and ventral side were pinned down, and the dorsal abdominal plate removed. The digestive tract was teased aside to access the reproductive organs, specifically looking at the development stage of the oocytes. Development stage of oocytes was assessed by a ranking system from 1 to 5 designed by Ann Nielson (Rutgers University), whereby rank 1 represents undeveloped oocytes with no more than one immature oocyte per ovariole, and rank 5

represents a post-vitellogenic female with distended ovaries and degenerating oocytes (Nielsen et al., 2017). This ranking system is based upon a more traditional 9 rank system by Katayama et al. (1993).

Statistical analysis of mobility data

To assess the effect of temperature alone on the mobility of diapausing *H. halys*, time series plots were made of mobility counts and temperatures for the temperature simulation treatment. Regression analysis on these time series were performed (the dependent variable was the mobility counts series and the explanatory variable was the temperature series). Autocorrelations in residuals of this regression analysis was tested with the Ljung-Box Q statistic and found statistically non-significant (p= 0.119). Therefore, the regression analysis was appropriate to determine the relationship between the mobility counts and temperatures and no further time series analysis was required.

4.2.4 Volatile compounds released by dead *Halyomorpha halys*

Ten *H. halys* were taken from the diapausing cohorts, individually placed in sealed glass 36 ml tubes, and freeze-killed overnight. They were stored in a temperature control chamber alongside the temperature simulation treatment described in 4.2.3 for the length of a journey from Baltimore, USA to Auckland, New Zealand (26 days). The headspace from the glass tubes was sampled before placing them in the CT chamber, and twice weekly from then on. Samples were collected using SuperQ VCTs at 400 ml/min for 10 minutes. Samples were extracted, stored and analysed as in 4.2.2. A system blank comprising an empty sealed tube kept alongside the treatment tubes was taken using the same apparatus and extraction technique for each time point. Resulting chromatograms were used to identify any compounds present. The quantities of each compound detected in the initial time sample were calculated against the internal standard using ratio response factors (RRF). For full RRF calculations see Appendix B (B.1).

4.3 Results

For access to chromatographic data see Appendix C.

4.3.1 Simulation of motion

Of the target compounds, 4-oxo-*(E)*-2-hexenal was never detected, and tridecane was found in all blanks, therefore both were removed from data analysis. Thus, target compounds used were *(E)*-2-decenal and dodecane. For *(E)*-2-decenal, no statistically significant differences in occurrences were found between samples and controls at any time point: T1 (FET, $p = 1.00$), T2 (FET, $p = 0.99$), T3 (FET, $p = 0.99$), T4 (FET, $p = 1.00$), T5 (FET, $p = 0.99$). For dodecane, no statistically significant differences in occurrences were found between samples and controls at any time point: T1 (FET, $p = 0.99$), T2 (FET, $p = 0.193$), T3 (FET, $p = 1.00$), T4 (FET, $p = 1.00$), T5 (FET, $p = 1.00$).

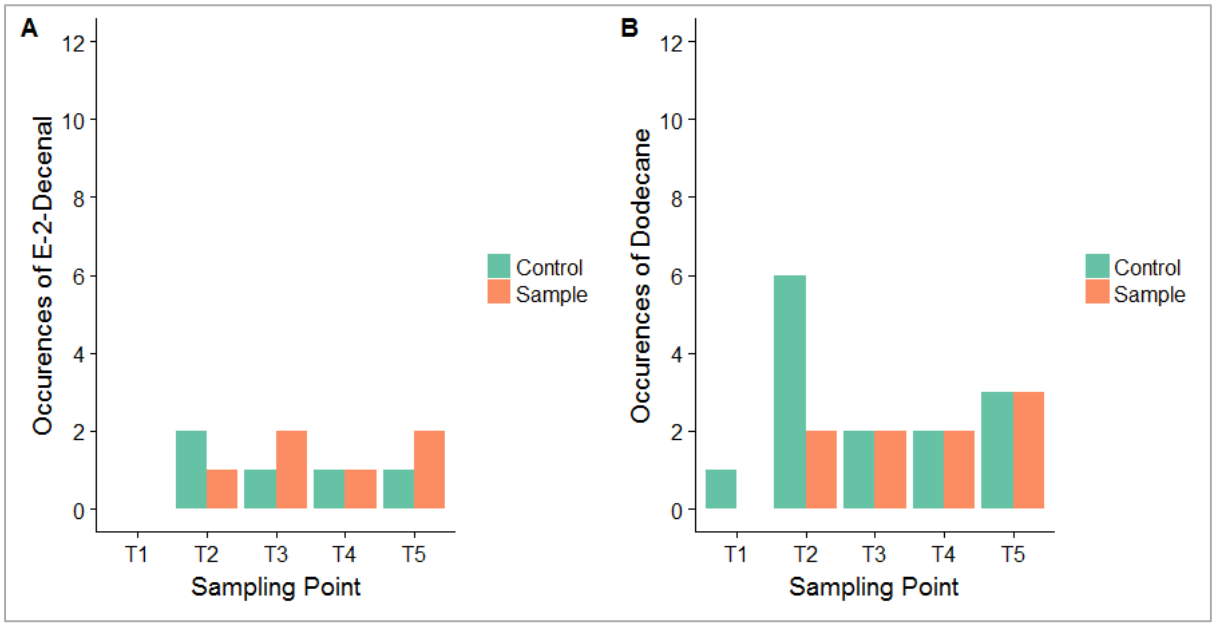

Figure 4.5 The number of occurrences of two target compounds A) *(E)*-2-decenal and B) dodecane in diapausing *Halyomorpha halys* populations undergoing movement simulation (*n*=12) and control populations remaining stationery (*n*=12). No significant differences in occurrence between samples and controls (Fisher's exact test, $p > 0.05$).

Simulated motion over this time period had no statistically significant effect on mobility (between sample and control populations, FET, $p > 0.05$) or mortality (zero bugs dead in samples or controls).

4.3.2 Simulation of temperature changes

No mobility was observed in any populations of *H. halys* kept in refrigerated and ambient temperature populations over the 26 day treatment period. Figure 4.6 shows the average number of *H. halys* that were mobile when counted every 2 – 3 days during the 26 day temperature simulation. As time progressed and the temperatures increased, the average count of mobile bugs steadily rose to the peak of 17.6 at 30°C and 16 days into the simulation. The first decrease in mobile bug counts was 19 days into the simulation at 30°C; the average mobility counts continued to decrease to 1.1 by day 26. Regression analysis showed a positive relationship between temperature and number of mobile *H. halys* (p = 0.009) within the temperature simulation treatment.

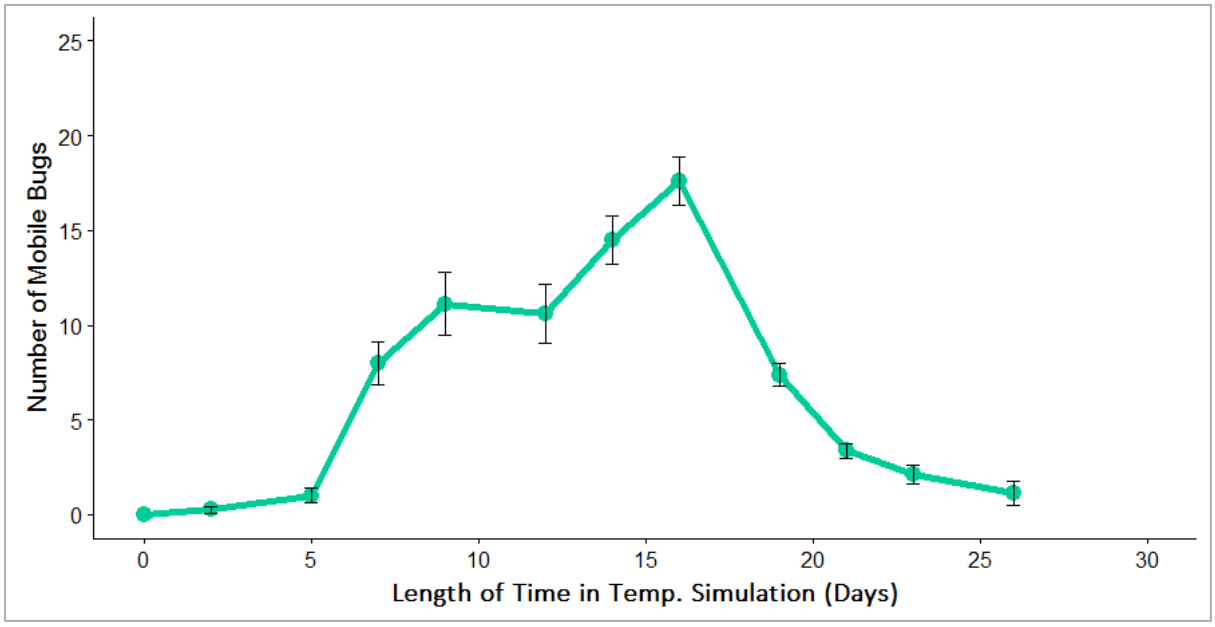

Figure 4.6 Line graph showing the counts of mobile *Halyomorpha halys* at 2 – 3 day intervals during a 26-day simulation of temperatures experienced on a trans-Pacific cargo ship. Points represent mean counts of eight populations ± *SEM*.

No mortality was observed in the populations kept in ambient temperatures. On average 0.37 ± 0.24% population mortality was observed in the refrigerated populations. An average of 89.5 ± 4.0% population mortality was observed in populations undergoing the temperature simulation treatment.

No mobility was observed in the refrigerated and ambient temperature populations on the final day. Of the remaining live bugs in populations undergoing the temperature simulation treatment 31.3 ± 15.6% were mobile, 50% of the populations contained mobile bugs (range 1 -5 bugs) and the remaining had zero mobile bugs on the final day.

All dissected females removed from the temperature simulation treatment on day 26 and those taken from non-experimental diapausing cohorts were found to be rank 1 in oocyte development stage. All oocytes were undeveloped.

4.3.3 VOCs released by dead *H. halys*

Table 4.1 The number of occurrences of *Halyomorpha halys* defensive compounds taken from the headspace of dead/decomposing *H. halys* over three weeks.

Compound	**Occurrences at sampling time (*n*=10)**			
	0 weeks	1 week	2 weeks	3 weeks
Tridecane	10	9	8	8
(E)-2-decenal	9	4	4	5
4-oxo-*(E)*-2-hexenal	9	2	5	3
Dodecane	6	5	1	1

The average quantities per dead bug of the four major compounds at 0 weeks were 6.45 µg of tridecane (RRF 0.68), 2.43 µg of *(E)*-2-decenal (RRF 0.16), 1.18 µg of 4-oxo-*(E)*-2-hexenal (RRF 0.71), and 0.035 µg of dodecane (RRF 4.8).

There were also minor compounds detected in samples, all of which were found less than a total of five times. These were decanal (in three samples at week 2), tetradecane (in two samples at week 1), a 13C unknown (in two samples at week 2), and pentadecane (in one sample at week 1).

4.4 Discussion

Simulated shipping movement did not increase the likelihood of volatile emission from diapausing *H. halys* populations; volatiles were detected in <15% of overall populations. Thus, broadly, these results do not support the idea of using these volatiles as the basis for a detection tool for *H. halys*, at least over the short amount of time that this simulation was conducted. Unfortunately, logistics precluded the experiment being extended beyond five days per round. It is therefore possible that closer to the 26-day voyage period, there may be higher levels of volatile accumulation. Also, it seems that volatile accumulation may occur in stationary populations of *H. halys* in a confined space. This study showed that bugs in stationary control boxes were just as likely to produce volatile profiles as those undergoing simulated movement. In Chapter 2, it was shown that non-agitated aggregations of diapausing *H. halys* left for seven days for VOCs to accumulate produced profiles mainly consisting of tridecane and *(E)*-2-decenal; the results seen in the current experiment likely come from the same accumulation with the conditions applied here having no effect. All of this would suggest that the simulated ship motion was not a sufficient level of agitation to induce the release of defence compounds. As the aggregations were settled into secure spaces of their own volition, as they would be in a real-life shipping scenario, the movements were not enough to make them feel endangered.

There was a significant effect of temperature on the number of mobile *H. halys* in a diapausing population. Statistical analysis shows that this effect is independent of the time variable. The pattern of bug mobility (Figure 4.6) and the high mortality rates observed on the final day suggest that the *H. halys* that became mobile had died rather than revert to diapause immobility. This was almost certainly due to a lack of food and moisture. *Halyomorpha halys* tend to prioritise foraging as they emerge from overwintering sites. European populations have been observed coming out of

overwintering sites as early as January during mild winters (Costi, Haye, & Maistrello, 2017). This generally culminates in those bugs dying as there are no actively growing crops at that time of year to feed on.

The dissected dead female bugs showed that they had neither become sexually mature, nor mated. This would suggest that temperature is responsible for disrupting the *H. halys'* settled diapause state, but not a strong enough factor to trigger an end to reproductive diapause. Nielson's work has shown that photoperiod is the main contributor to bringing *H. halys* out of diapause, with 21 days out of diapause and feeding necessary for the onset of oviposition (Nielsen et al., 2016; 2017). All treatments in the temperature simulation study were kept in the dark, removing the photoperiod variability triggering the end of diapause. It would therefore be interesting to repeat the study with foraging material available to the bugs and see if this influences reproductive diapause without extended photoperiod, and how much it would affect mortality. It is believed that it was already-mated females entering the USA which began all east coast populations (Valentin et al., 2017; Xu et al., 2014). Given the results of this study have shown no mating, it is reassuring that it would be improbable for establishing *H. halys* populations to hitchhike into the Southern Hemisphere.

As noted above, the high mortality rates found after the 26-day temperature simulation show that any volatiles released by dead and decomposing *H. halys* would be valuable in aiding VOC detection of aggregations of these bugs. The compounds reported in Table 4.1 are all as reported in Chapter 2 (see 2.3.2), with alkanes dominating the profile, and aldehydes making a large contribution. Once more, the most abundant profile is that of the four defensive compounds identified in Chapter 2: tridecane, (*E)*-2-decenal, 4-oxo-(*E)*-2-hexenal, and dodecane. This suggests that the VOC profile is produced by the decomposition of the bug's scent gland, located on the dorsal surface of the abdomen. This four compound profile can therefore be indicative of both agitated live *H. halys* and aggregations including dead bugs, which this study would suggest is highly likely after a trans-Pacific journey.

4.5 Conclusion

The robot simulation technology could be a valuable tool for further study of *H. halys* behaviour providing data on mobility, mortality, and defensive behaviours. The same is also likely to apply to other potential pest species. The simulation methodology can undoubtedly produce information that is valuable for improving the monitoring and management of invasive hitch-hiking species generally. More specifically, if similar technology could be acquired for the sole purpose of such experiments, then it may be possible to repeat this study on *H. halys* for the extended 26-day time period, as confirmation of what has been found here. The fact that dead/decomposing *H. halys* release VOCs for weeks is heartening, and theoretically, with highly sensitive detectors, could contribute to screening for the detection of *H. halys* in shipping containers.

Further work investigating diapausing *H. halys* responses to temperature differences is likely to be important in both planning biosecurity monitoring work and as a management input for dealing with *H. halys* in those countries that have already been invaded. It has been suggested that understanding and finding overwintering aggregations could play a substantial role in population control (Toyama et al., 2011). Additional studies that can be done would include the mentioned repetition of this experiment but with the addition of foraging material. The temperature simulation could also be repeated with populations housed in the metal shelters described both here and in Chapter 2, whereby VOC samples are collected at time points as the bugs begin to warm up and become mobile.

Chapter 5

Determination of Instrumental Limits of Detection for Analysis of Volatiles Released by *Halyomorpha halys* and Suitability for Detection within Large Contained Spaces

5.1 Introduction

The analysis of biogenic volatile organic compounds (BVOCs) requires instrumentation capable of separation of complex mixtures, specific compound identification, and quantitative results comparable to reference materials (Dewulf & Van Langenhove, 2011). Gas chromatography – mass spectrometry (GC-MS) is widely used for analysis of BVOCs for these reasons. Separation by GC can be used for acquisition of a general chromatogram containing all compounds within an unknown BVOC mixture; manipulation of parameters (temperature programme, column flow etc.) can be used to improve peak resolution if necessary. Detection by MS can be used in total ion count (TIC) mode to qualify all compounds present in a mixture. To simplify complex chromatograms and improve sensitivity, MS can also be run in selected ion monitoring (SIM) mode; this mode only scans for ions specified in the method, and can be set to detect ions unique to or abundant in the analytes being targeted. GC-MS can work with a wide range of concentrations whilst maintaining high sensitivity, making it ideal for the analysis of trace amounts of target compounds (Santos & Galceran, 2003). GC-MS is still commonly used as a benchmark method for newer techniques: when Henderson et al. (2010) investigated the use of a portable E-nose for the detection of stink bugs, GC-MS was used to confirm the identity of the volatiles being detected. GC-MS has been the ideal analytical instrument for the work in this thesis: identification of unknown ranges of VOCs and reliable quantitation of compounds in set small volumes.

Much of GC-MS' sensitivity for analysis of trace amounts relies upon sampling steps with high pre-concentration of analytes and/or high recovery rates. The volatile collection trap (VCT) loaded with SuperQ is classed as an active sampler, as the hand pump pulls air through the adsorbent filter. A study

on pepper weevil (*Anthonomus eugenii* Cano) pheromones used a similar sampling procedure using SuperQ VCTs as described in Chapters 2 through 4, and found SuperQ to have a 98% recovery rate when sampling from a 44 ml glass tube (Eller & Palmquist, 2014). It was also found that SuperQ was suitable for sampling a wide range of insect pheromones. SuperQ is a porous polymer adsorbent, and is commonly used for plant and insect volatile collection, as is a Tenax, an adsorbent filter of similar material. Other commercially available active samplers have been tested for the recovery rates of 49 VOCs classed as indoor air quality pollutants, including numerous alcohols and ketones (Miyake, Tokumura, Wang, Wang, & Amagai, 2017). The three samplers compared contained either petroleum or coconut shell based adsorbent materials, and were found to have an average recovery rate over the 49 compounds of 88% (range 78 – 94%). The purpose for studying those samplers was for the use of detecting levels any of the 49 VOCs within occupational settings, e.g. car-manufacturing plants, and therefore relevant to the application of sampling within large volumes.

Previous chapters have discussed the VOCs released by diapausing *Halyomorpha halys* Stål, the amounts they are released in, and the biological and ecological context of such emissions. Overall, it would appear that diapausing bug aggregations release either all of or a subset of the defensive VOC profile comprising tridecane, *(E)*-2-decenal, 4-oxo-*(E)*-2-hexenal, and dodecane. The overarching question to ask here, is whether or not these compounds and this emission profile are detectable within large enclosed volumes such as shipping containers, and what the detection limits are of conventional analytics using GC-MS.

5.2 Methods and materials

5.2.1 Optimising Gas Chromatography – Mass Spectrometry Method

All GC-MS methods described below were analysed on a Shimadzu GCMS2010 (Ultra) with an RTX-5MS column (30 m x 0.25 mm I.D.), with GCMSsolutions software. Auto-sampling was performed by the PALS LHX-xt system.

5.2.2 Preparation of calibration standards

Standards were all prepared from tridecane, *(E)*-2-decenal, *(E)*-2-hexanal, and dodecane (all >94%, Sigma-Aldrich, Australia). Unfortunately, 4-oxo-*(E)*-2-hexenal was unavailable in New Zealand, and it was found that suppliers were unable to ship to New Zealand with packaging appropriate for conserving this highly volatile compound. In its place, *(E)*-2-hexenal was used as a proxy. This proxy is appropriate to the extent that both 4-oxo-*(E)*-2-hexenal and *(E)*-2-hexenal are compounds found in heteropteran scent glands and utilised as defensive secretions, as such both are of low molecular weight and highly volatile (Farine, Bonnard, Brossut, & Le Quere, 1992). The addition of an oxygen gives 4-oxo-*(E)*-2-hexenal a larger molecular weight, therefore the *(E)*-2-hexenal proxy will elute at a shorter retention time than its counterpart. This additional oxygen will also make 4-oxo-*(E)*-2-hexenal more reactive, and therefore more prone to degradation than either its proxy or any of the other three VOCs found in the profile. Each of five calibration levels contained the four compounds at concentrations with relative abundances approximate to those stated in Chapter 2 (Table 2.1). The concentration of each compound in the five levels, along with calculated relative abundances are presented below in Table 5.1. To each calibration level standard was added the internal standard tetralin (1,2,3,4-tetrahydronaphthalene) at a concentration of 4.04 μg/μl.

Table 5.1 The concentrations of each compound in the *Halyomorpha halys* defence odour VOC profile (tridecane, *(E)*-2-decenal, *(E)*-2-hexenal as a proxy for 4-oxo-*(E)*-2-hexenal, and dodecane) as found in prepared calibration standards at five concentration levels, and the calculated abundance % of each.

	Compound concentrations, µg /µl			
Calibration level	Tridecane (49%)	*(E)*-2-decenal (27%)	*(E)*-2-hexenal (22%)	Dodecane (2%)
1	1.58	0.88	0.71	0.06
2	3.15	1.75	1.41	0.13
3	4.73	2.63	2.12	0.19
4	6.30	3.50	2.82	0.25
5	8.51	4.38	3.53	0.31

5.2.3 Total ion count/ scan method

The calibration level 2 standard was analysed in triplicate using the TIC method as per previous chapters (see 2.2.6and 3.2.4); this method was derived from that used by Solomon et al. (2013).

The GC-MS method used a high pressure 1 µl splitless injection at an injection temperature of 250°C. The GC was operated at a column flow of 0.6 ml/min. The temperature programme started at 40°C for 7 minutes, followed by temperature ramping of 6°C/min until a final temperature of 230°C was reached and held for 5 minutes. The mass spectrometer was run in total ion count mode, with a scanning range 25-550 m/z, with source temperature at 250°C and quad at 150°C.

5.2.4 Selected ion monitoring

The GC method above was maintained. The mass spectrometer was run in SIM, detecting ions 43, 57 and 71 for tridecane and dodecane, 41, 55, and 70 for *(E)*-2-decenal, 41, 55, and 69 for *(E)*-2-hexenal, and 91, 104, and 132 for tetralin (IS). The calibration level 2 standard was analysed in triplicate on the SIM method.

5.2.5 Increased injection volume

The SIM GC-MS method above was maintained, however, the high pressure injection volume was increased to 2 µl. The calibration level 2 standard was analysed in triplicate on the 2 µl SIM method.

5.2.6 Limits of detection and quantitation calculations

The set of five calibration standards, run in triplicate, was analysed using the 2 µl SIM GC-MS method described above. A calibration curve for each compound using the internal standard peak area ratios was constructed for use in calculations to determine the limits of detection (LoD) and quantitation (LoQ). For calibration curves see Appendix A (A.2). The LoD is defined as the lowest concentration of an analyte at which an instrument, in this case GC-MS, can be reported as detecting said analyte. The LoQ is the lowest concentration at which an instrument can detect the analyte at a level reliable enough to report its quantity rather than just presence. For dynamic systems such as GC, the standard deviation of residuals, also known by root mean square error, approach for calculating LoDs and LoQs is considered reliable and easy to measure (Bernal, 2014). For the determination of limits of detection and quantitation, the standard deviation of the residuals for each analyte was calculated using equation 1.

Equation 1

$$s = \sqrt{\frac{\sum(yi - \bar{y})^2}{n - 2}}$$

s= standard deviation of the residuals

yi =predicted instrumental response for analyte concentration

$\bar{y}$= actual mean of instrumental responses for analyte concentration

n= number of calibration levels

The instrumental response for the limits of detection and quantitation for each analyte were calculated using equations 2a and b.

Equation 2a

$$yLoD = c + 3s$$

Equation 2b

$$yLoQ = c + 10s$$

c= calibration intercept

y= instrumental response

s= standard deviation of the residuals

These instrumental limits were applied to the linear equation from each calibration to calculate the LoD analyte concentrations for each analyte. The LoDs are the more appropriate limits to follow in this case, as the desired outcome is to be able to report detection of these compounds, leaving quantities somewhat irrelevant. However, if concentrations are found to be above the LoDs, then quantities could be used to estimate aggregation sizes.

5.2.7 Theoretical calculations for detecting *Halyomorpha halys* in container scenarios

The information gathered in Chapters 2 and 4 on the amounts of each VOC in the *H. halys* defensive odour profile released by agitated or dead bugs can be used to calculate whether an active sampler and GC-MS system could be used for detection of *H. halys* in shipping containers. Equation 3 was used to calculate whether each analyte would be detectable in this system.

Equation 3

$$\left(\frac{(Amt_{com} \times N_b)_{live} + (Amt_{com} \times N_b)_{dead}}{Vol}\right) \times RR \geq LoD_{com}$$

Amt_{com}: amount of selected compound released per bug, µg

N_b: number of *H. halys* bugs in scenario

Vol: volume of container, l

RR: recovery rate of active sampler

LoD_{com}: GC-MS limit of detection for selected compound, µg/µl

The numbers of bugs, both alive and dead, were taken from information on aggregations of *H. halys* intercepted by MPI at the New Zealand border in recent years (Cath Duthie, MPI, personal communication). The volume of container used for this calculation was based upon a standard size (20-foot) shipping container offered by a commercial company (https://www.mrbox.co.uk/shipping-containers/) i.e. 38,000 litres. The recovery rate of active samplers consists of an average rate taken from the aformentioned study (see 5.1) comparing three commercially available active samplers used to sample 49 VOCs for the evaluation of indoor workplace air quality and was set for these calculations at 88% (Miyake et al., 2017). The calculations that were made assumed that i) all live *H. halys* are releasing defensive odours, ii) all dead *H. halys* have been dead less than one week, iii) there is an even distribution of VOCs throughout the container. These assumptions lean towards predictions assuming the highest concentrations within solvent extractions and sampler recoveries, and don't necessarily account for further dilutions.

Below are three scenarios taken from the aforementioned MPI interception data, these scenarios include the highest numbers of *H. halys* intercepted.

- Scenario 1: An aggregation of 26 living and 20 dead *H. halys*.
- Scenario 2: An aggregation of 49 dead *H. halys*.
- Scenario 3: An aggregation of 36 live *H. halys*.

For full calculations of concentrations found in each scenario see Appendix B (B.2).

5.3 Results and Discussion

5.3.1 GC-MS method optimisation

As expected, the SIM GC-MS method with an increased injection volume was the most sensitive compared to the other GC-MS methods tested (Figure 5.1). This method was therefore used to analyse

the calibration standards and calculate the limits of detection and quantitation. Injection volumes for this kind of GC methods can theoretically be increased further than 2 µl in order to increase peak area for analytes. In practice, increased injection volumes tend to cause peak broadening. The beginnings of peak broadening can be observed in Figure 5.1 for the tetralin, dodecane, *(E)*-2-decenal, and tridecane peaks, whereby the bases of the peaks of the 2 µl injection are wider than those peaks from the 1 µl injections. The *(E)*-2-hexenal peak at both injection volumes displays broadening and tailing, suggesting that the standard for this compound has already started to deteriorate, but it is much more prominent in the peak from the 2 µl injection.

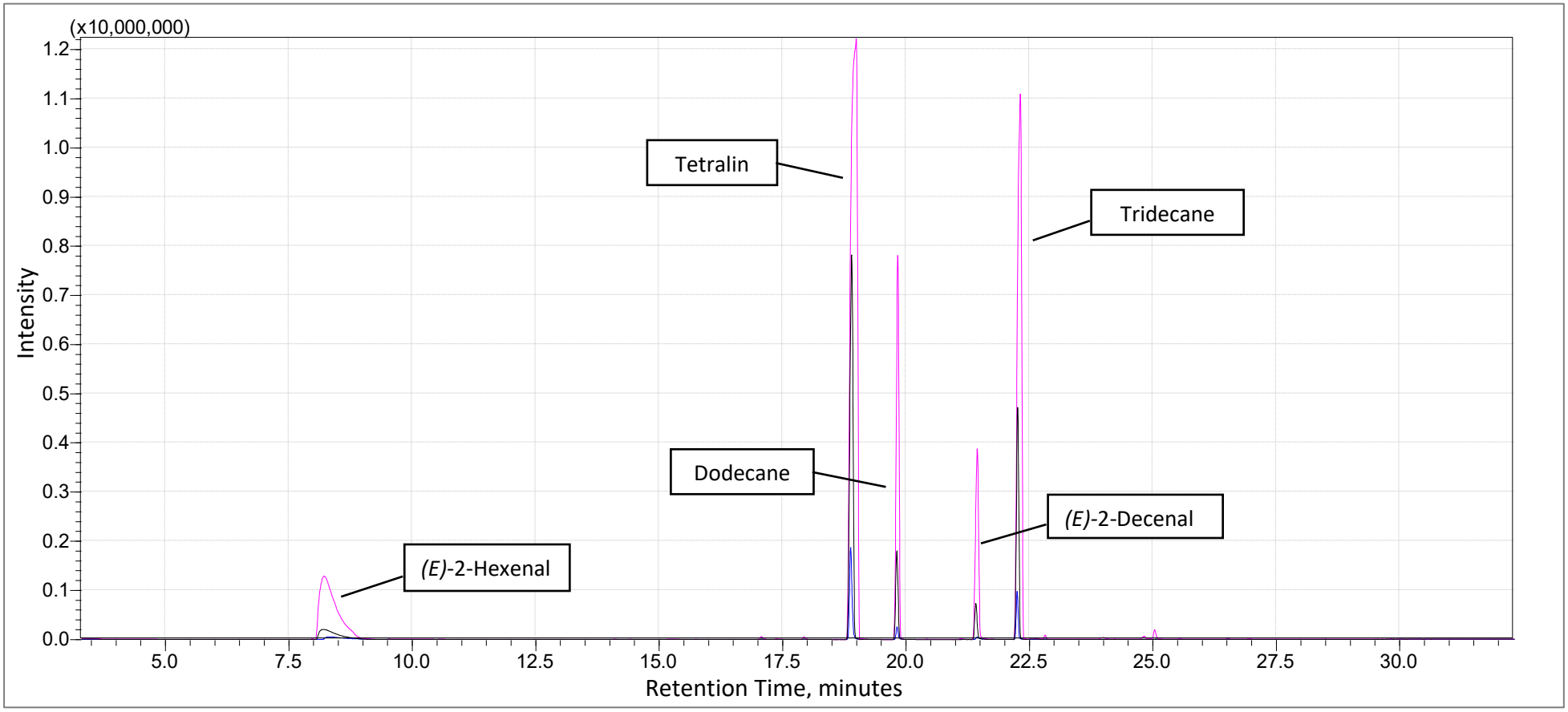

Figure 5.1 Comparative chromatogram showing GC-MS methods used to analyse a low-level calibration standard mix of the compounds released by agitated *Halyomorpha halys*: total ion count (blue), selected ion monitoring (black), and selected ion monitoring with 2 µl sample injection (pink).

The limits of detection calculated using the standard deviation of residuals from the calibration curve were 0.562 µg/µl for tridecane, 1.154 µg/µl for *(E)*-2-decenal, and 0.013 µg/µl for dodecane. The *(E)*-2-hexenal standard was not distinguishable from noise in most of the resulting chromatograms, and was therefore excluded from data analysis. For 4-oxo-*(E)*-2-hexenal calculations following, an average of these three limits of detection was used, therefore 0.0576 µg/µl.

5.3.2 Theoretical detections of *Halyomorpha halys* in container scenarios

The scenarios presented here are very high-end estimates of what would be found in realistic container scenarios. The bug numbers used for calculations are taken from the largest reported aggregations, and the assumptions presented don't consider lack of air flow or any escape of VOCs. Even with these exaggerated circumstances, the system of using an active absorbent sampler and analysis on GC-MS would not be suitable for detections of *H. halys* VOCs in such large volumes. None of the scenario figures calculated in Table 5.2 fall within the limits of detection of this system.

Table 5.2 The concentrations of each compound found in the *Halyomorpha halys* defensive odour profile which would be present in samples collected, using an active sampler to be analysed on a GCMS, in three theoretical scenarios. Scenario 1 is an aggregation of 26 living and 20 dead *H. halys* in a standard shipping container (38,000 l). Scenario 2 is an aggregation of 49 dead *H. halys* in a standard shipping container. Scenario 3 is an aggregation of 36 living *H. halys* in a standard shipping container. All scenario results calculated using equation 3, and instrumental limits of detection for GC-MS analysis of each compound provided for reference.

	Compound concentrations, µg/l			
	Tridecane	*(E)*-2-decenal	4-oxo-*(E)*-2-hexenal	Dodecane
Limit of Detection	5.62×10^{7}	1.15×10^{6}	1.30×10^{8}	5.76×10^{8}
Scenario 1	0.028	0.012	0.010	0.001
Scenario 2	0.007	0.003	0.001	<0.001
Scenario 3	0.035	0.015	0.013	0.001

Gas chromatography-mass spectrometry is a well-established method for the detection of volatiles, however sensitivity for this method depends very much on sample collection and pre-concentration steps. This can pose a challenge with very large headspace samples, therefore more novel techniques which rely less on this would be more appropriate for this work. Direct injection mass spectrometry (DIMS) methods, such as selected ion flow (SIFT)-MS and proton-transfer-reaction (PTR)-MS, are able to analyse ambient air samples in real time and cut out the need for an extraction step (Biasioli, Yeretzian, Märk, Dewulf, & Van Langenhove, 2011). Both mentioned techniques use soft ionisation methods to minimise fragmentation of sample ions, reducing the number of ion overlaps on the resultant mass spectrum, which negates the need for chromatographic separation whilst still allowing

complex VOC mixtures to be analysed in real-time (Smith & Španěl, 2011). Although SIFT-MS is most commonly known for its uses in medical breath analyses and other human metabolic processes, studies illustrating its uses in analysis of environmental gas samples produced by combustion engines, animal waste, and food industry have shown SIFT-MS' diverse range (Smith & Španěl, 2005). SIFT-MS was successfully used to detect phosphine with no sample preparation at linear concentrations ranging parts per million (ppm) to parts per trillion (ppt), and for this analyte the LoD identified for a 10 s scan was 0.27 pg/ml (Milligan, Francis, Prince, & McEwan, 2007). Considering scenario 2, as the scenario with the lowest concentration of VOCs, then three of the four compounds from the defensive odour profile would be found in a container above this LoD, tridecane at 8.32 pg/ml, *(E)*-2-decenal at 3.13 pg/ml, and 4-oxo-*(E)*-2-hexenal at 1.52 pg/ml, with only dodecane at 0.04 pg/ml falling below detection. PTR-MS can be used for similar analyses types to SIFT, and has been used for VOC collections of fruit emissions and decaying biomatter (Lindinger, Hansel, & Jordan, 1998; Lindinger & Jordan, 1998). This technology is also reported to detect at ppt levels. Overall, DIMS technology seems to be a strong contender for use in *H. halys* VOC detection in containers, if developed and applied to this use. Electronic noses have also been developed to detect pre-programmed VOC profiles in headspace. These devices benefit from being portable, they pull headspace from the area over carbon black-polymer composite sensors which are trained to a specific profile (Lampson, Khalilian, Greene, Han, & Degenhardt, 2014). Studies have shown e-noses to exhibit up to 100% accuracy in detecting heteropterans, such as *Nezara viridula* Linnaeus and *Megacopta cribraria* Fabricius, and damage caused by such species to host crops (Henderson et al., 2010; Lampson, Degenhardt, Greene, Khalilian, & Han, 2017; Lampson et al., 2014). The disadvantages being that this device is still under recent development, it has been found to decrease in accuracy from 24 hours after training the sensors, and LoDs for VOCs for this sensor sits around the 10 ppb range, with the most conservative reports claiming sub-ppm levels (James, Scott, Ali, & O'hare, 2005; Lampson et al., 2014; Santonico et al., 2012). Were e-nose technology able to reach the LoDs reported for DIMS technology, it would ideal for *H. halys* detection, as it is fit for purpose in many other practical ways.

5.4 Conclusion

The technology available to this project, i.e. GC-MS coupled with SuperQ VCT samplers, is not sufficiently sensitive for use as a *H. halys* VOC detector within containers. The predictive detections calculated for this system aren't realistic, as there are far more variables than can be easily predicted and quantified. What this predictor has shown, is that laboratory testing of this system may not be worth time and resources. However, there are technologies available that could be repurposed which already reach the desired levels of VOC detection. Laboratory trials which mimic *H. halys'* release of VOCs would be useful in conjunction with such technology as e-noses or direct injection mass spectrometers.

Overall, it has been shown that a detectable VOC profile is released by aggregations of diapausing *H. halys*. However, detection experiments would require more sensitive technology than was available to this project.

Chapter 6

General Discussion and Concluding Comments

The volatile organic compound (VOC) profiles for species of stink bugs have been well studied and reported. However, the establishment of a full odour volatile organic compound profile for *Halyomorpha halys* Stål had not been found in literature prior to the beginning of this project. Another vital piece of information for the pursuit of this project was to ascertain whether or not *H. halys* in diapause would emit their defensive odour. As extensively discussed in Chapter 1, and throughout, diapausing aggregations of *H. halys* are of significant concern for Southern Hemisphere border biosecurity. Therefore, the first prominent finding in this thesis was that *H. halys* in the diapause state, does indeed release defensive odours. These odours were elicited when the bugs were agitated. The VOC profile under this condition has been established as follows (in order of abundance): tridecane, *(E)*-2-decenal, 4-oxo-*(E)*-2-hexenal, and dodecane. Further, the headspace VOCs taken from bug aggregations which were left in a non-agitated and settled state were still found to comprise a volatile profile consisting of tridecane and *(E)*-2-decenal. These findings exhibit the potential for the premise of detection of *H. halys* via the VOCs they release, as the diapausing bugs are capable of releasing VOCs both actively and passively. This information demonstrates the suitability of *H. halys* as a model species for this study.

With this knowledge, it was essential to determine the cues which would trigger *H. halys* to release the more substantial defensive VOC profile. Preliminary observations made during the defensive VOC collections in Chapter 2 suggested that *H. halys* required the presence of another bug during agitation to elicit a defensive odour response. Therefore, the cues for *H. halys* to release their defensive odours were investigated in relation to the bug being present in groups rather than individually. This showed that agitation within a group causes a high likelihood of odour release, suggesting that the aggregatory behaviour of diapausing *H. halys* would actually facilitate high likelihoods of defensive odour release. Also, it was found that an individual bug exposed to one component of the odour, 4-oxo-*(E)*-2-hexenal, acts as a trigger to elicit its own defensive odour. Therefore, it was recognised that should one bug in

an aggregation release odours, there would be a cascading effect as others would similarly release. As *H. halys* are commonly found in aggregations within containers, rather than as individuals, this signal amplification is significant in improving the likelihood of detecting *H. halys* by their defensive VOCs rather than relying on the two-compound passive profile. As well as confirming this amplification, it was verified that mechanical agitation is the prominent inducer of defensive odour release. However, this is focussed only to those variables that are likely to be encountered within the shipping scenario and precludes predation, foraging, and mating situations etc.

Simulation experiments were also conducted during this project in terms of the detection of *H. halys* VOCs resulting from shipping movement. The work extended previous chapters' experiments that had involved "high mechanical agitation" to elicit defence responses (i.e., the experimenter vigorously shaking the vessel containing the bug/s) by more realistic simulation of the agitation which would be experienced by bug aggregations within a shipping containers at sea and during port handling. It was prudent to ascertain whether or not this is sufficient to elicit defensive odours. Additional observations were also made during this simulation as to the effects on mobility and mortality. The study suggested that shipping movement does not have a significant effect on diapausing *H. halys* in terms of VOC release, mobility, or mortality. Though this simulation was run over a short period of time, as discussed in Chapter 4's discussion, these VOC measurements may have differed if the simulation could have lasted for the extent of a Northern to Southern Hemisphere ship voyage (e.g. 26 days), as passively emitted VOCs would have had more time to accumulate.

A longer term simulation was also run to investigate the effects of temperature on the mobility and mortality of diapausing aggregations of *H. halys*. The high mortality rate observed in the populations undergoing the fluctuating temperatures is thought to be down to the high mobility, found to be caused by increasing temperatures. There was no moisture or foraging material available to the bugs on becoming mobile, and they therefore probably starved to death. As this simulation had a significant effect on diapausing *H. halys'* activity, it would be worth exploring this temperature variable further. As suggested in Chapter 4, repeating this experiment but settling populations into metal shelters, and

taking VOC samples throughout the simulation may produce a more realistic insight into how *H. halys* VOC patterns may be affected over the course of a sea journey. The high mortality rate observed in the fluctuating temperature simulation also led to VOC collection from dead bugs. This showed that aggregations of dead *H. halys* as they degrade would actually be releasing the same VOC profile as agitated bugs, but in smaller quantities.

The system for VOC detection used throughout this thesis was active samplers containing SuperQ absorbent material, eluting samples, and analysing them on GC-MS. This is known to be a sensitive and reliable system for detecting a wide range of VOCs, and was fit for the purpose of analysing headspace collections over the small volumes and high densities of *H. halys* tested during experiments in Chapters 2 through 4. However when theoretically applied to the large volume of a standard container with the sizes of *H. halys* aggregations found during border biosecurity checks in New Zealand, this system would not be capable of detecting *H. halys* presence. Even the most high-end, conservative estimate of concentrations of *H. halys* VOCs that could be present was magnitudes from the limit of detection. While the configurations used in this thesis were not all optimum for definitively determining the biosecurity value the VOC approach for *H. halys* detection, there is strong evidence that with additional resources, the work could be vastly expanded. A laboratory study which finds a way to either mimic the release of *H. halys* VOCs in different states (passively, actively, or through degradation) or can reliably use aggregations of the insects would be of immense value. Practical investigations into the persistence of the four VOCs (tridecane, *(E)*-2-decenal, 4-oxo-*(E)*-2-hexenal, and dodecane) after release from the bugs would be important to knowing if a detection of VOCs system would be viable practically. For instance, if the two VOCs most indicative of stink bug presence, *(E)*-2-decenal and 4-oxo-*(E)*-2-hexenal, were to degrade too quickly for border inspectors to test the containers, then this process would be impractical. Predictions in Chapter 5 are made under the assumption that any VOCs released are evenly distributed, which is unrealistic in a system of stagnant air which is most likely in a container situation. Therefore, were a sensitive enough analytical process to be established, an additional study using said system with VOCs in an actual container would be relevant to establish the effect of air flow on detection processes.

What this study has accomplished is the identification of the volatile profiles being emitted by non-agitated groups of *H. halys*, agitated groups of *H. halys*, and dead *H. halys*. A combination or sub-set of these profiles would be present in a container, in which diapausing *H. halys* are present and has undergone/is undergoing a long ship journey. Although the analytical system used in this study was not sensitive enough for the process of detecting these profiles to indicate the presence of *H. halys*, it has established foundations for further highly targeted investigation.

The use of simulating shipping journey variables could be an invaluable tool for furthering biosecurity monitoring efforts of this, and other invasive species, at the border. Knowledge of the mobility, mortality, and other biological behaviours can be used in threat assessment and to improve inspection methods for particular insect species.

Appendix A

Quantitative Calibration Graphs

A.1 Quantitative calibration graphs for Chapter 2

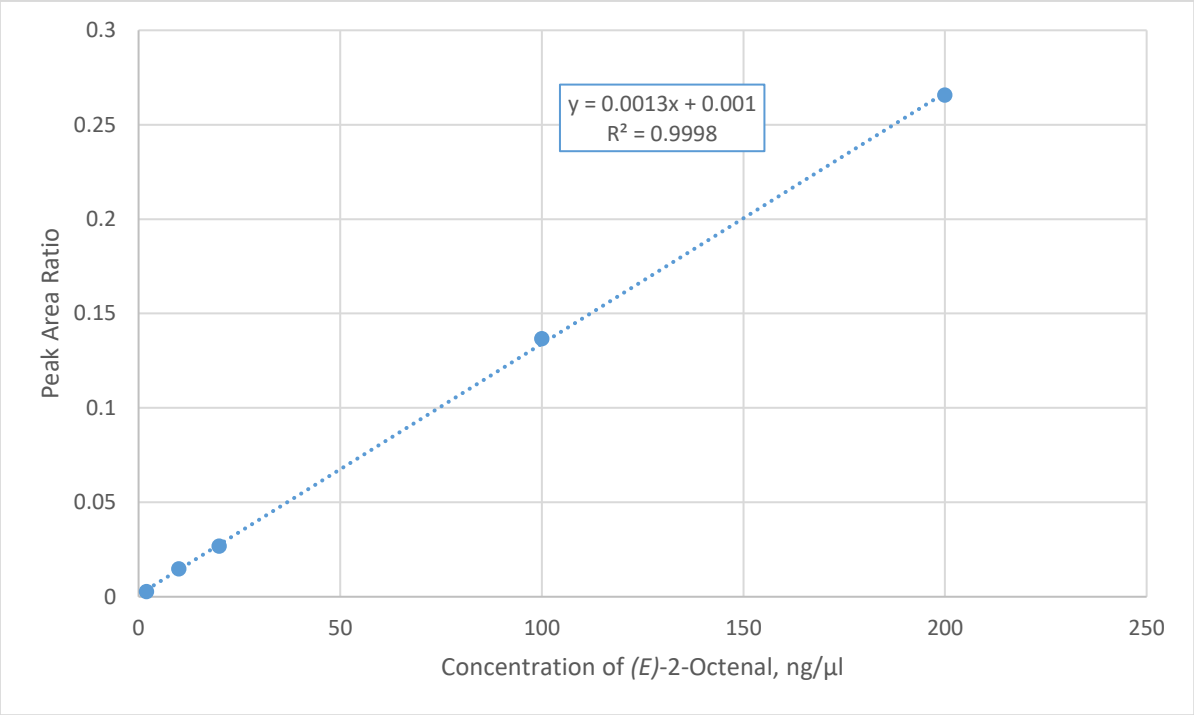

Figure A. 1 Quantitative calibration graph constructed from standard concentrations of *(E)*-2-octenal, range 2 – 200 ng/µl, analysed on GC-MS with tetralin as an internal standard at 200 ng/µl.

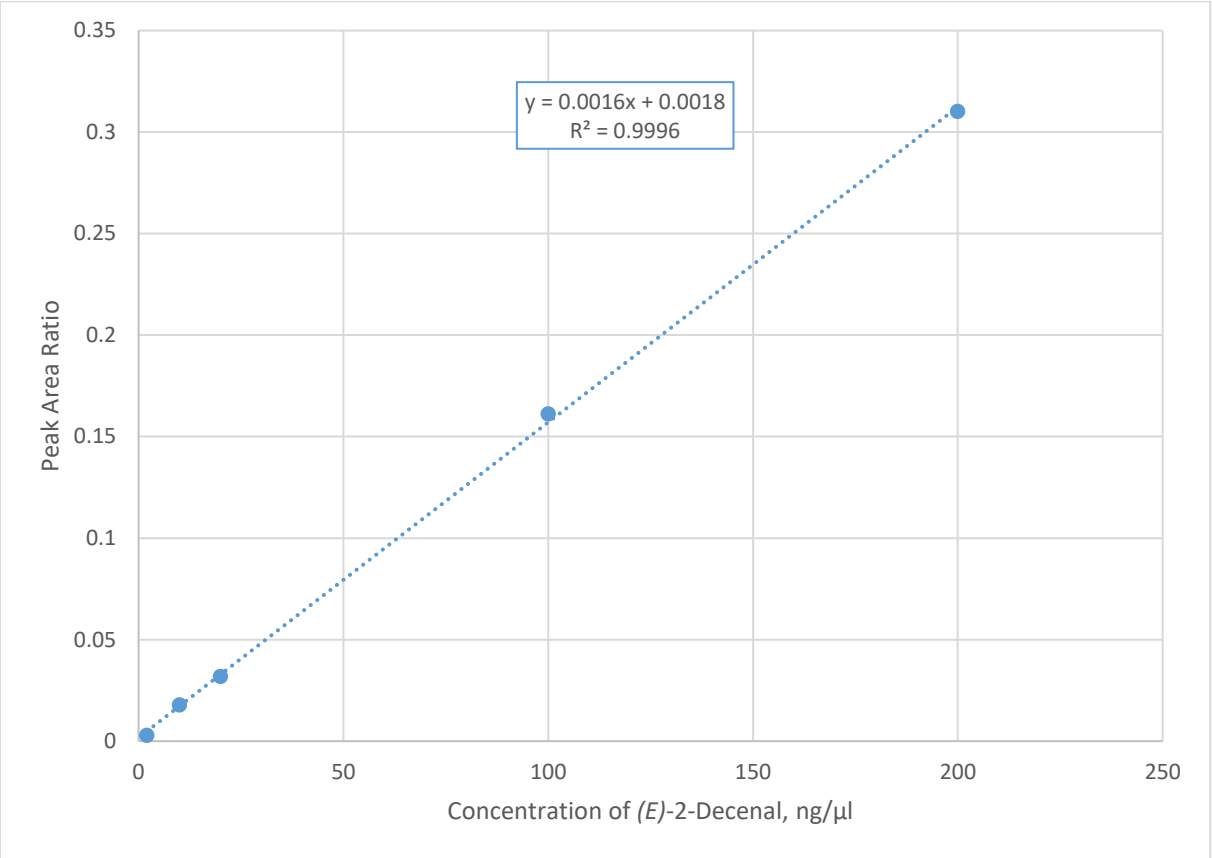

Figure A. 2 Quantitative calibration graph constructed from standard concentrations of *(E)*-2-decenal, range 2 – 200 ng/µl, analysed on GC-MS with tetralin as an internal standard at 200 ng/µl.

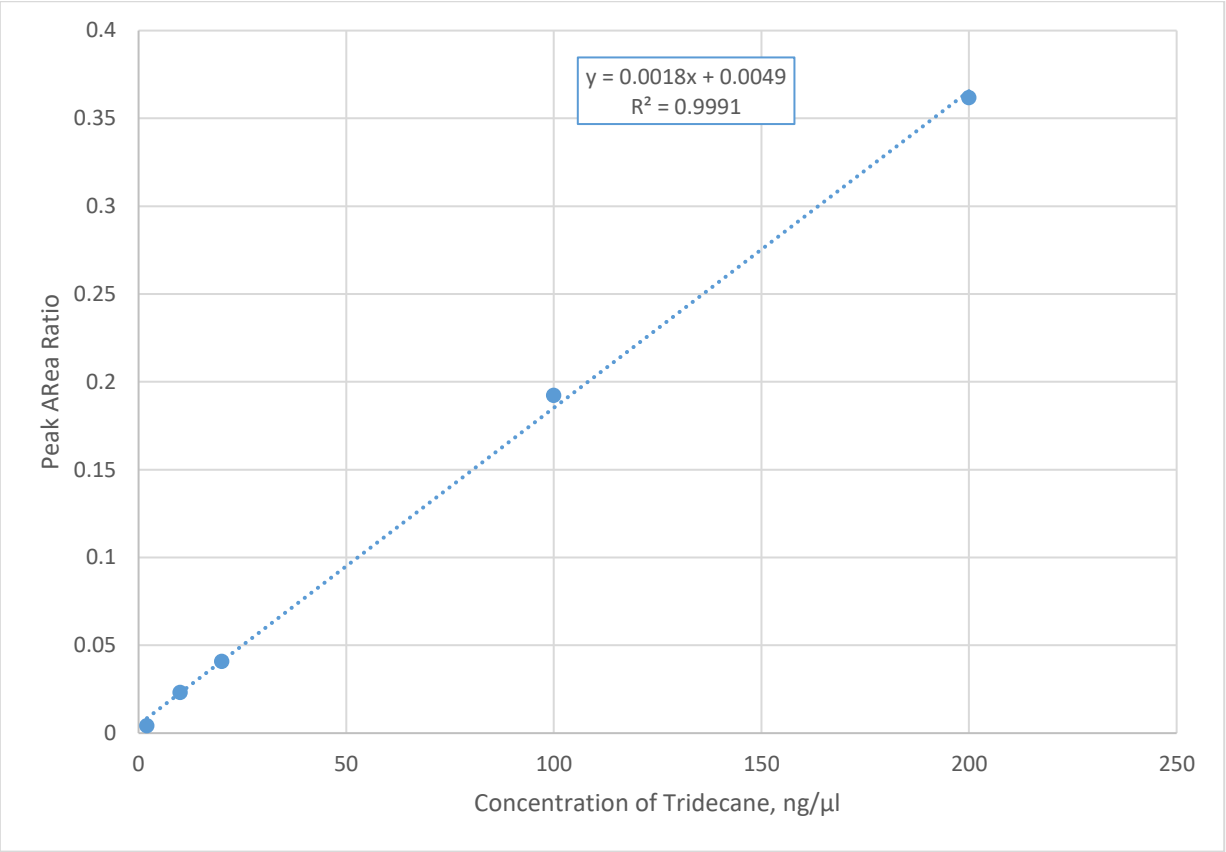

Figure A. 3 Quantitative calibration graph constructed from standard concentrations of tridecane, range 2 – 200 ng/µl, analysed on GC-MS with tetralin as an internal standard at 200 ng/µl.

A.2 Calibration graphs for Chapter 5 limit of detection calculations

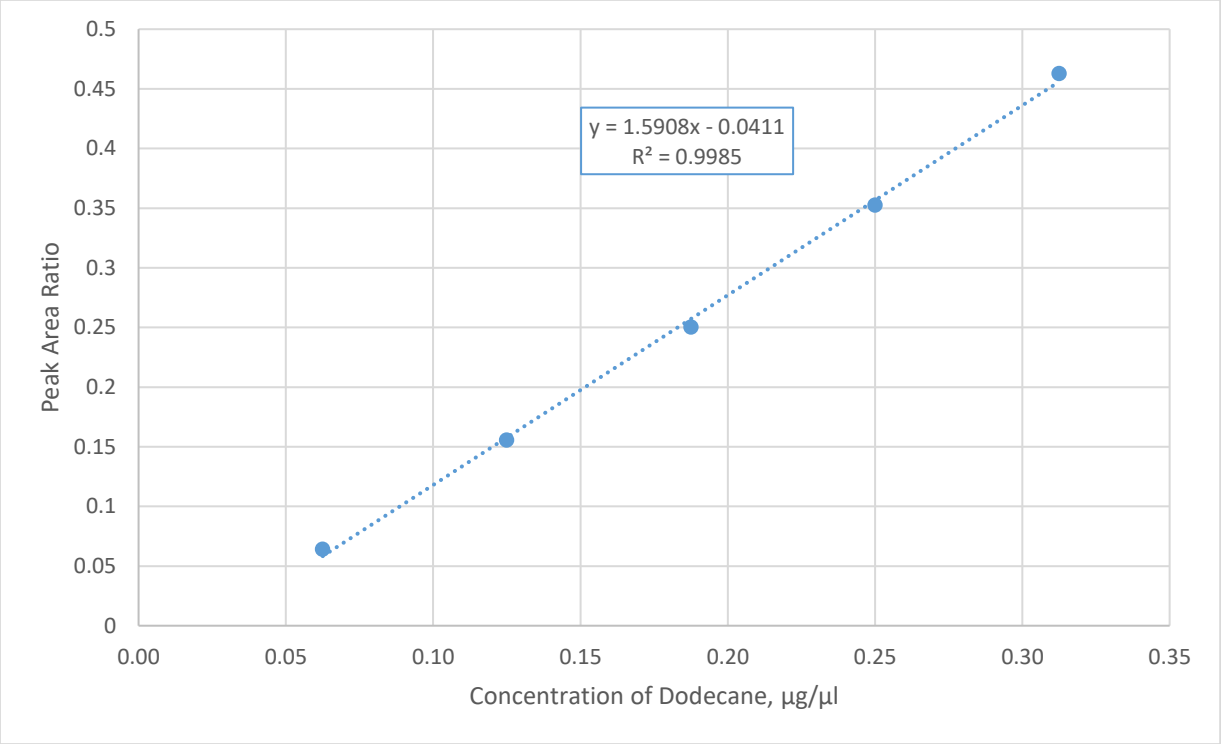

Figure A. 4 Quantitative calibration graph constructed from standard concentrations of dodecane, range 0.06 – 0.31 µg/µl, analysed on GC-MS with tetralin as an internal standard at 4.04 µg/µl.

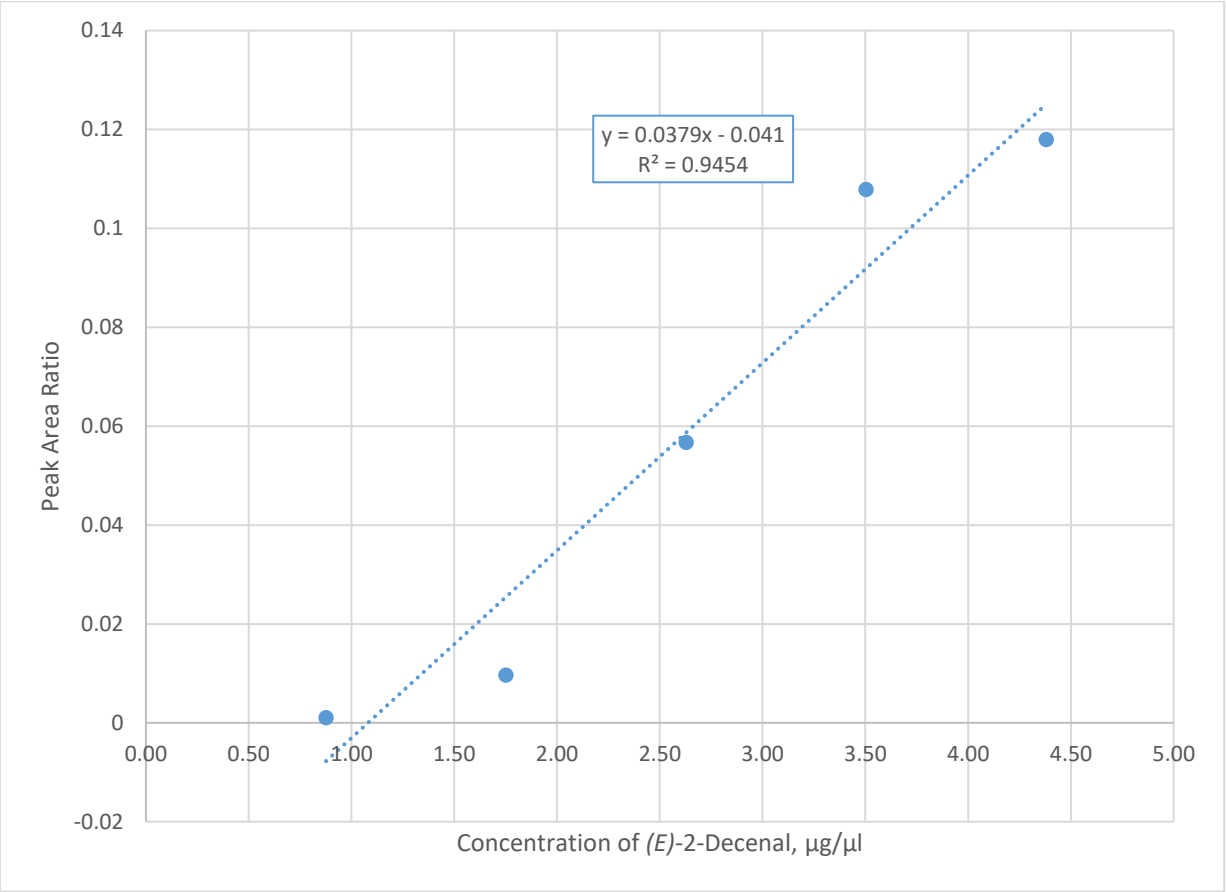

Figure A. 5 Quantitative calibration graph constructed from standard concentrations of *(E)*-2-decenal, range 0.88 – 4.38 µg/µl, analysed on GC-MS with tetralin as an internal standard at 4.04 µg/µl.

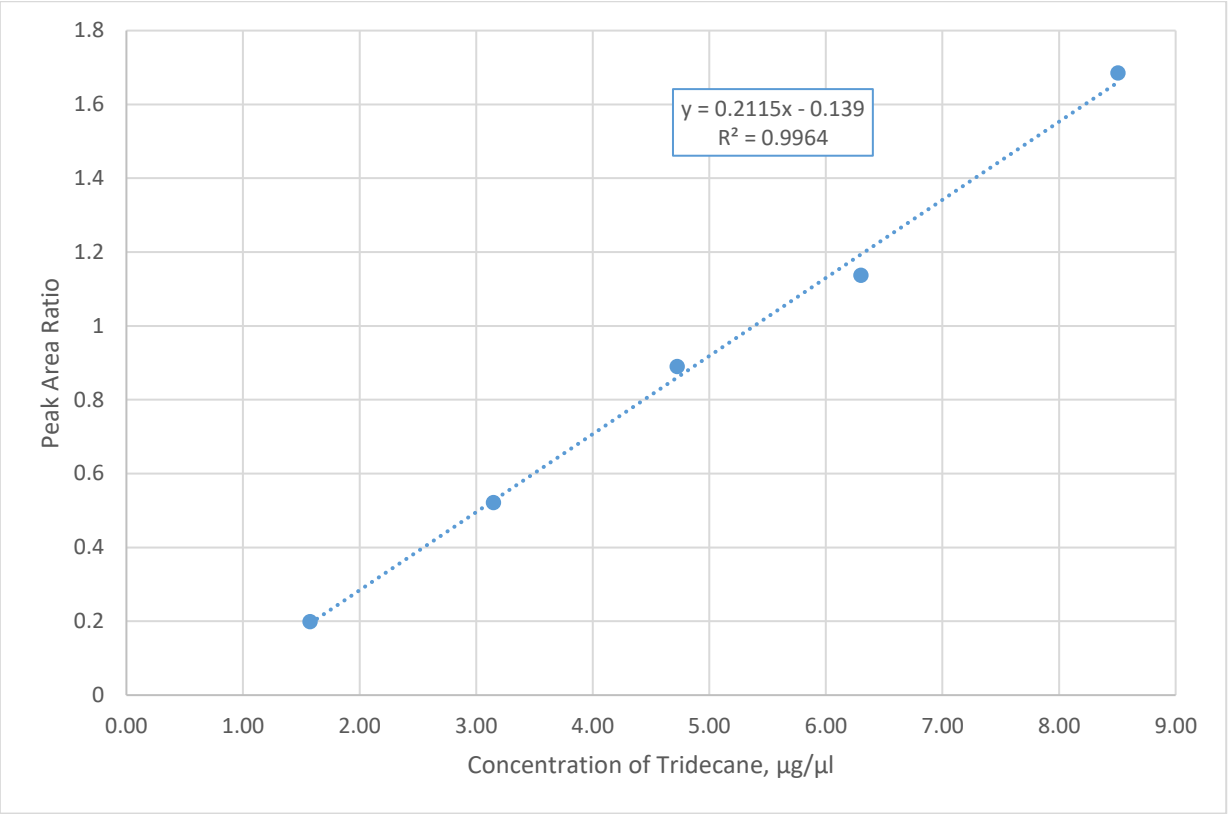

Figure A. 6 Quantitative calibration graph constructed from standard concentrations of tridecane, range 1.58 – 8.51 µg/µl, analysed on GC-MS with tetralin as an internal standard at 4.04 µg/µl.

Appendix B

Additional Calculations

B.1 Quantitative data for Chapter 4 dead bug VOCs with relative response factor

Equation for calculating relative response ratio from standard calibration chromatograms with an internal standard:

$$RRF = \frac{(PA_{com} \times Conc_{IS})}{(Conc_{com} \times PA_{IS})}$$

RRF= relative response factor

PA_{com}= peak area of analyte compound

$Conc_{IS}$= concentration of internal standard

$Conc_{com}$= concentration of analyte compound

PA_{IS}= peak area of internal standard

Equation for calculating unknown concentrations of analytes using the RRF:

$$Conc_{com} = \frac{(PA_{com} \times Conc_{IS})}{(RRF \times PA_{IS})}$$

Table B 1 **Amounts of defensive compounds released from individual, dead Halyomorpha halys immediately following freeze-killing, as calculated using relative response factor with internal standard, tetralin.**

Repeat	**Quantity of Compound, µg**			
	Tridecane	*(E)*-2-Decenal	4-Oxo-*(E)*-2-Hexenal	Dodecane
1	17.09	1.64	1.60	0.05
2	9.98	2.20	1.18	0.02
3	3.09	1.12	0.53	n.d.
4	7.27	3.69	1.22	0.03
5	7.32	5.36	2.04	0.05
6	1.44	0.45	0.35	n.d.
7	6.77	5.22	1.39	0.04
8	4.54	1.18	1.20	0.02
9	2.85	1.05	1.14	n.d.
10	4.11	n.d.	n.d.	n.d.
Average	6.45	2.43	1.18	0.03

B.2 Calculations of concentrations of *Halyomoprha halys* defensive VOCs according to theoretical scenarios presented in Chapter 5

B.2.1 Scenario 1: An aggregation of 26 living and 20 dead *Halyomorpha halys*

Tridecane released from aggregation

$$\left(\frac{(41.7 \times 26) + (6.45 \times 20)}{38000}\right) \times 0.88 = 0.028\ \mu g/l$$

(E)-2-decenal released from aggregation

$$\left(\frac{(18.2 \times 26) + (2.43 \times 20)}{38000}\right) \times 0.88 = 0.012\ \mu g/l$$

4-oxo-(E)-2-hexenal released from aggregation

$$\left(\frac{(15.8 \times 26) + (1.18 \times 20)}{38000}\right) \times 0.88 = 0.010\ \mu g/l$$

Dodecane released from aggregation

$$\left(\frac{(1.5 \times 26) + (0.03 \times 20)}{38000}\right) \times 0.88 = 0.001\ \mu g/l$$

B.2.2 Scenario 2: An aggregation of 49 dead *Halyomorpha halys*

Tridecane released from aggregation

$$\left(\frac{6.45 \times 49}{38000}\right) \times 0.88 = 0.007\ \mu g/l$$

(E)-2-decenal released from aggregation

$$\left(\frac{2.43 \times 49}{38000}\right) \times 0.88 = 0.003\ \mu g/l$$

4-oxo-(E)-2-hexenal released from aggregation

$$\left(\frac{1.18 \times 49}{38000}\right) \times 0.88 = 0.001\ \mu g/l$$

Dodecane released from aggregation

$$\left(\frac{0.03\ \times 49}{38000}\right) \times 0.88 = < 0.001\ \mu g/l$$

B.2.3 Scenario 3: An aggregation of 36 living *Halyomorpha halys*

Tridecane released from aggregation

$$\left(\frac{41.7\ \times 36}{38000}\right) \times 0.88 = 0.035\ \mu g/l$$

(E)-2-decenal released from aggregation

$$\left(\frac{18.2\ \times 36}{38000}\right) \times 0.88 = 0.015\ \mu g/l$$

4-oxo-(E)-2-hexenal released from aggregation

$$\left(\frac{15.8\ \times 36}{38000}\right) \times 0.88 = 0.013\ \mu g/l$$

Dodecane released from aggregation

$$\left(\frac{1.5\ \times 36}{38000}\right) \times 0.88 = 0.001\ \mu g/l$$

Appendix C

Digital Access to Data

All chromatograms, and raw peak area data for Chapter 2: Identification of Volatiles Released by Diapausing Brown Marmorated Stink Bug, *Halyomorpha halys* (Hemiptera: Pentatomidae) has been made available on open access database Figshare. To access please see: figshare.com, DOI 10.6084/m9.figshare.5056906.

All chromatograms and EthoVision data for Chapter 3: *Halyomorpha halys* Group Behavioural Responses to Chemical and Tactile Stimuli has been made privately available on online database Figshare. To access please see: https://figshare.com/s/a504e03baa4da6ad34f7; DOI 10.6084/m9.figshare.5643154.

All chromatograms for Chapter 4: Brown Marmorated Stink Bug: A Simulated Voyage has been made privately available on online database Figshare. To access please see: https://figshare.com/s/9853cfd03023ba0053cc; DOI 10.6084/m9.figshare.5643169.

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